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BIOLOGICAL BULLETIN

EXPERIMENTAL TRANSFORMATIONS OF BIPOLAR FORMS IN *CORYMORPHA PALMA.*

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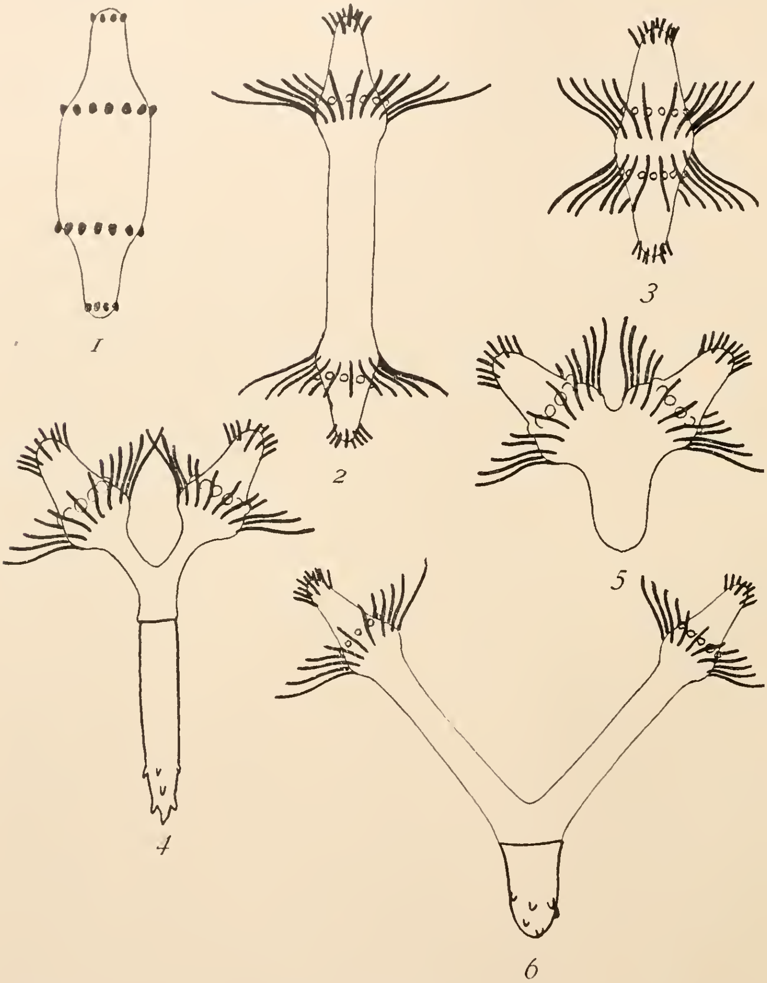
It has been shown in earlier papers that the localization and development of a basal end in the reconstitution of stem pieces of *Corymorpha* may be determined by the dominance of more distal parts (Child, '26*b*, '27*c*), by contact with or nearness to the bottom (Child, '26*b*, '27*a*, *b*) and by the inhibiting action of various agents (Child, '27*a*, *b*, *c*). In normal development the base arises from the low end of the primary gradient (Child, '26*a*), a region of relatively low metabolism, but contact with, or nearness to the bottom and various inhibiting agents, or both acting together, may determine basal ends anywhere, even as mosaic parts on other body regions (Child, '26*b*). Evidently these external factors induce physiological conditions similar to those which arise in embryonic development at the low end of the primary gradient. The present paper gives the results of a few experiments on the transformation of bipolar forms resulting from reconstitution into other forms, its purpose being merely to record the occurrence of such transformations under certain experimental conditions. All figures are drawn from observed and recorded cases. Regions inclosed in perisarc are indicated by heavier line than naked regions and perisarcal accumulations not in direct contact with the body surface are indicated by dotting. Except in case of the very small individuals, the figures are diagrammatic as regards numbers of tentacles.

TRANSFORMATION OF BIPOLAR INTO BIPOLAR-UNIPOLAR FORMS.

Bipolar forms are those in which a hydranth develops from both distal and proximal end of a stem piece. As shown elsewhere (Child, '26*b*), bipolar forms may range from extreme apical ends including merely mouth and hypostome, to complete hydranths with a considerable length of stem between them. Bipolar forms of the sort used in these experiments are shown in Figs. 1–3. Fig. 1 is an earlier, Fig. 2 a later stage of a form with considerable length of stem between the two hydranths, such as ordinarily develops from the longer stem pieces. Fig. 3 shows



Figs. 1–6. Bipolar and bipolar-unipolar forms resulting from reconstitution of stem pieces.

a bipolar form from a shorter piece, consisting of hydranths only. Bipolar-unipolar forms (see Child, '26*b*) are bipolar distally and unipolar proximally (Figs. 4–6). The two hydranths develop from the two cut ends of the piece, the unipolar proximal region from the lateral stem region between the hydranths. The proximal region represents a new polarity at right angles to the original.

By means of inhibiting conditions it is possible to transform bipolar forms such as Figs. 1–3 into bipolar-unipolar forms like Figs. 4–6, although the bipolar forms never develop a unipolar proximal region as long as they are kept under good conditions Extensive experimentation along this line has not been undertaken, but several agents have been used, viz., alcohol, two and three per cent., NH_4Cl $m/300$ and ethyl urethane $m/300$ and $m/100$ and in case of one lot of pieces sea water which had stood for some hours in a new galvanized bucket was found very effective as an inhibiting agent. The same transformations appeared in all agents used. The bipolar forms were placed in the agent at earlier (Fig. 1) or later stages (Figs. 2, 3) of development, but only after it was certain that they were developing as symmetrical bipolar forms.

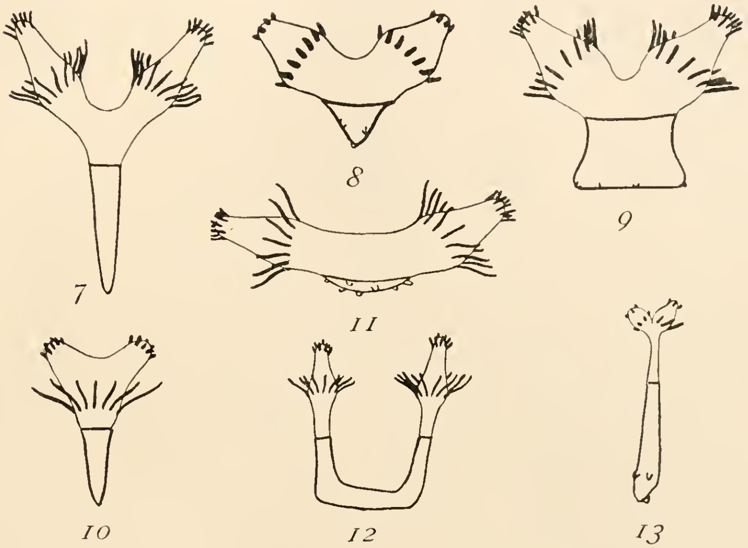
In most cases they remained in the agent one to two days and were then returned to sea water. One series, however, remained in alcohol two per cent. for twelve days, the solution being renewed daily. During exposure to the agent the tentacles are usually reduced to mere stumps or disappear entirely, the distal regions in most cases disintegrating and the stumps undergoing resorption. Hydranths and the stem if it is present, undergo marked reduction in size, particularly in the longer exposure periods and in some cases the manubrium of one or both hydranths may show some disintegration. On return to water the pieces remain undisturbed for two days or more and during this time redevelopment of hydranths and tentacles takes place. If the degree of inhibition is not too extreme the basal region may develop during exposure to the agent, otherwise it develops after return to water.

Fig. 7 shows the transformation of a bipolar form like Fig. 2 after alcohol two per cent. for one day, then alcohol three per cent. for a second day. The figure represents a stage three days after return to water. A proximal stem region and basal end have developed from what was originally the lateral stem region between the hydranths. A similar case is shown in Fig. 8. This form, originally bipolar like Fig. 2 but somewhat shorter, was in NH_4CL $m/300$ one day then in sea water two days. At this time redevelopment of tentacles and development of the new base were not far advanced, as Fig. 8 indicates. Fig. 9 was transformed from a piece like Fig. 1 after forty hours in alcohol two per cent. followed by two days in sea water. In this series the pieces were subjected to inhibition at a relatively early stage of the bipolar development and the new basal end was larger in all than when later stages were used.

In cases in which the degree of inhibition is somewhat greater, either because of higher individual susceptibility or because of higher concentration or longer exposure to the agent, modification of form may be more extreme. In the case of Fig. 10, for example the piece was originally bipolar like Fig. 2 but somewhat shorter. It was subjected to alcohol two per cent. for one day, then to alcohol three per cent. for a second day and then remained three days in sea water. As Fig. 10 shows, the proximal regions of the two hydranths have undergone fusion and instead of the two complete sets of proximal tentacles originally present, only one set now appears. In this case there has been not only reorganization of the lateral stem region into a base, but extensive reorganization of the hydranths has also occurred. Other similar cases have been observed.

Figs. 11 and 12 show two stages of the transformation in the toxic sea water. The form was originally bipolar like Fig. 1, but under the inhibiting conditions developed the basal region with holdfast outgrowths within twenty-four hours on the part in contact with the bottom. The basal region continued to grow at the expense of other parts and after four days the condition shown in Fig. 12 was attained. This case was not observed further, but forms of this sort usually separate sooner or later into two individuals, the separation occurring through the gradual atrophy of some portion of the connecting basal region. The region undergoing atrophy apparently becomes a sort of no-man's-land between the two individuals and its cells may be at one time influenced by one, at another time by the other and perhaps sometimes by neither. This absence of definite function probably determines the atrophy.

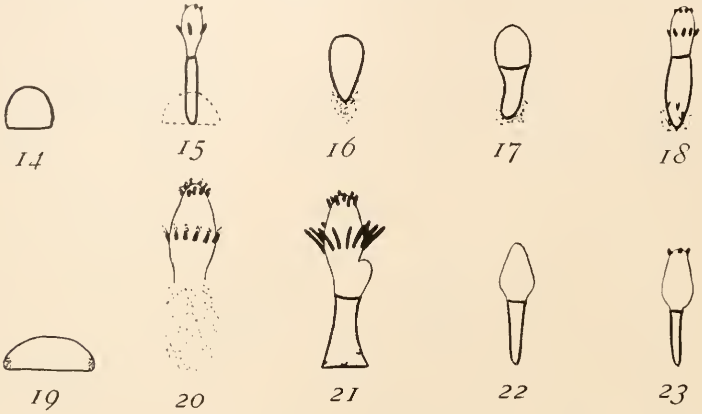
The case shown in Fig. 13, originally bipolar like Fig. 2 was in alcohol two per cent. for twelve days. During this time it decreased greatly in size, largely because of disappearance of the axial parenchyma, the tentacles disappeared completely and the form of the hydranths approached that of early developmental stages. After return to water redevelopment of the two hydranths began and a basal tip developed from the side of the stem in contact, underwent elongation and became disproportionately large at the expense of other parts. Fig. 13 shows the condition seven days after return to water. It will be noted that the hydranth on the left appears to be more inhibited than the other. As will appear below, the two hydranths of a bipolar form often show a considerable difference in susceptibility.



Figs. 7–13. Bipolar-unipolar forms transformed from bipolar forms by inhibiting conditions.

Transformation of Bipolar into Unifolar Forms.

The first experiments on subjection of bipolar forms to inhibiting conditions were performed in 1910 and it was found that some bipolar forms after several days under inhibiting conditions redeveloped as completely unipolar forms. Work had to be discontinued before much experiment along this line was possible, but the observations made on the changes occurring during the inhibition period showed in some cases complete disappearance of the more highly specialized structures and the gradual regression of the form to a rounded mass, usually more or less completely inclosed in perisarcal secretion (Fig. 14). After return to water a single apical region developed on the upper surface of this mass and a complete new axis vertical to the substratum resulted (Fig. 15). Such cases were believed to represent the obliteration of the double polarity of the bipolar form and the determination of a new polarity by the differential of position, *i.e.*, the differential between the upper free surface and the surface in contact with



Figs. 14–23. Development of unipolar from bipolar forms determined by inhibiting conditions. Figs. 14, 15. Apparent obliteration of both original polarities by inhibiting conditions and determination of new single polarity by the differential of position. Figs. 16–18. Unipolar from bipolar forms by death of one and persistence of other axis. Fig. 19. Equal regression under inhibiting conditions of both hydranths of a bipolar form: the hypostome regions indicated as dotted areas. Fig. 20. Death and disintegration under inhibiting conditions of one axis of a bipolar form with persistence of other. Fig. 21. A form in process of becoming unipolar by death of most of one axis and development of a base from lateral stem region. Figs. 22, 23. Unipolar forms from small fragments of bipolar forms which remained alive while rest was killed by inhibiting conditions.

the bottom which supposedly determines a difference in rate of oxygen consumption and discharge of CO_2 and so initiates a new gradient (Child, '26*b*). A case of this sort was described and figured (Child, '15, pp. 144–6, Figs. 75–78; '24, p. 119 and Figs. 106–109). It was also noted in 1910 that some of the bipolar forms became unipolar during the inhibition period. This unipolarity was observed only after the pieces had undergone considerable reduction and was indicated merely by the somewhat elongated form tapering toward one end and an accumulation of perisarc at the slender end (Figs. 16, 17). With long periods of inhibition, *e.g.*, from twelve days to three weeks, such unipolar forms, whether derived from bipolar forms or from the reduction of unipolar forms, might undergo reduction to a length of less than one millimeter and still give rise to normal individuals after return to water (Fig. 18).

At the next opportunity in 1922 to continue these experiments the tank water at the laboratory was apparently somewhat toxic and there was difficulty in keeping material in good condition long enough for such experiments, consequently no further information on these transformations was obtained at that time. In 1924 and 1926 experiments were made with water taken direct from the ocean and it was found that some bipolar forms, more commonly the shorter, underwent regression without actual disintegration of any parts except the two sets of tentacles. Fig. 19 shows a case of this sort in which the two hypostome regions are still distinguishable by their whitish color at the two ends of the piece. They are indicated in the figure as dotted areas. In pieces returned to water at this stage redevelopment of the two hydranths with a new base between them occurred. When the inhibition was continued all indications of the original axes disappeared, although the diameter of the mass might be greater in the direction of the original axes. Such masses may give rise to unipolar forms after return to water and the polarity in such cases appears to be new and determined by the differential of position. But in 1924 and 1926 it was also found by more frequent observation of the material that one axis of a bipolar form is often more susceptible than the other and with a sufficient degree of inhibition it disintegrates, while the other remains alive (Fig. 20). The axis that remains alive may undergo regression to a condition like Figs. 16 and 17 and in such cases the polarity is one of the two polarities of the bipolar form, or when forms like Figs. 16 and 17 result from the reduction of whole stems or other unipolar forms, it is the original polarity. In such cases the bipolar form becomes unipolar through the death of one axis, rather than by the obliteration of the two original polarities and the determination of a single new polarity. Even in such cases, however, there is much reorganization. The hydranth appearing after return to water is not only absolutely but relatively very much smaller and has a much smaller number of tentacles than the original and the stem and base may develop from regions that were originally part of the hydranth.

The occurrence of this method of change from a bipolar to a unipolar condition is beyond question. It is not properly speaking, a transformation, but merely a persistence of an axis. Since it does occur, the question must be raised whether the apparent transformation by obliteration of the old axes and determination of a single new axis may not actually be merely the persistence of one of the axes. With respect to this point it may be noted, first, that in all cases of disintegration of one axis which have been observed the disintegration occurs early, before the hydranth has lost its form, and second, that both axes have been identified in other pieces after the form of the hydranth was indistinguishable and the only indication of the original axes which was externally visible was the hypostome regions which differed from the rest in color (Fig. 19). No disintegration has been seen in such cases at any later stage and some of them certainly give rise to single new polarities. To all appearances this single polarity is new and determined after obliteration of the old axes in these forms, but in the absence of landmarks after regression of the hydranths it is difficult to be entirely certain as to what happens in cases of this sort. It is hoped that there may be opportunity for further work in the future.

In the light of the data concerning the origin of new polarities in stem pieces (Child, '27*a*, *b*) there is no reason to doubt the possibility of obliteration of old and determination of new polarities in bipolar forms, particularly if some stem is present between the hydranths. Moreover the fusion of hydranths, their reorganization after regression and the development of basal ends from parts of the hydranth body under experimental conditions indicate that at least the more proximal levels of the hydranth possess considerable capacity for giving rise to other parts, particularly after some degree of regression.

Figs. 14–18 and 20–23 show observed cases of unipolar forms which developed from bipolar forms after subjection to inhibiting conditions. In Figs. 14 and 15 (twelve days in two per cent. alcohol) the single polarity is believed to be new. In Figs. 16, 17 and 18, also twelve days in alcohol, the polarity is almost certainly one of the original polarities, but it has developed proximal and basal regions at the expense of more distal parts. Fig. 19 shows equal regression of both axes, as already noted and Fig. 20 shows a case of disintegration of one axis and persistence of the other. Fig. 21, a bipolar form two days in water after forty hours in alcohol two per cent., is almost completely unipolar, but has a protuberance on one side, which represents the last traces of the other polarity and which later disappears. In this case the basal region has developed from the side of the piece, as in the bipolar-unipolar forms. That is, the unipolar condition has resulted from the death of the more distal regions of one axis and resorption of the remainder and the development of proximal region and base from the side of the stem. The unipolar form in Fig. 22 developed from a bipolar form forty-six hours in alcohol. When returned to water the piece was less than one millimeter in diameter and almost spherical and showed no trace of polarity. Three days later it had attained the stage shown in Fig. 22. Undoubtedly most of the original bipolar form had died, but it was impossible to determine what part of the form the small mass represented, though there is little doubt that it represented the less susceptible proximal region. The persistence of such minute fragments after death of the rest of the piece is of common occurrence under inhibiting condition and in all observed cases, whether they arose from bipolar forms or from stem pieces these masses develop, if they develop at all, as unipolar forms, the polarity apparently being determined by the differential of position. Fig. 23 shows another minute form which developed from a bipolar form after twenty-four hours exposure to NH_4Cl $m/300$ and two days in water. Here also a large part of the original form disintegrated, but in this case a polarity was evident at the end of the inhibition period, the form of the minute piece being similar to that of Fig. 16. In this case the final polarity undoubtedly represents one of the original polarities of the bipolar form, but completely reorganized.

To sum up: in the development of unipolar from bipolar forms all visible traces of the original polarities may disappear, regression to a rounded mass more or less completely inclosed in perisarc may occur and the axis of the unipolar form appears to be a new gradient determined by the differential of position. In other cases one of the axes of the bipolar form may die and disintegrate under the inhibiting conditions while the other, or some part of it remains alive. In such cases polarity is usually visible, at least in the form of the piece, at all stages and the final single polarity corresponds in direction with this polarity, though it represents extensive reorganization.

Discussion.

The numbers of cases in these transformation experiments are not large. Among forty bipolar forms resulting from reconstitution, subjected to inhibiting conditions sixteen (forty per cent.) transformed into bipolar-unipolar forms, ten (twenty-five per cent.) became unipolar, six (fifteen per cent.) remained bipolar and eight (twenty per cent.) died. This does not include preliminary experiments to determine proper concentrations nor experiments in which all pieces died. Numerous controls in water under good conditions have not shown such transformations in any case. The chief purpose thus far has been to establish the fact of these transformations rather than to determine their frequency. As regards the transformation of bipolar into bipolar-unipolar forms the data are clear and conclusive, but as regards the question of obliteration of both the original polarities and determination of a new single polarity as one method of the origin of unipolar from bipolar forms there is less complete certainty. The course of events in the cases recorded indicates that the two original axes are obliterated unless one resorbs the other after all visible traces of both have disappeared, but if they are so much alike physiologically that they undergo regression equally it is highly improbable that one is sufficiently different from the other to resorb it. Without resorption or death of one axis, there seems to be no escape from the conclusion that the two original axes are obliterated and a new one determined. That the original single polarity may be obliterated has been shown elsewhere (Child, '27*a, b*) and since this is possible there can be little doubt as to the possibility of obliteration of the double polarity of bipolar forms.

The question how the inhibiting conditions transform bipolar into bipolar-unipolar forms is answered very simply from the gradient standpoint. The middle region of the bipolar form where the two polarities meet represents the lowest physiological level present. The inhibiting conditions lower all levels to some extent and may bring the middle region to the level at which a basal region arises. It has already been noted that pieces from the naked stem region commonly secrete more or less perisarc under inhibiting conditions and may become completely inclosed in it (Child, '27*a*). Under normal conditions bipolar forms show almost continuous motor activity, elongation and contraction of the stem and movements of hydranth body and tentacles, consequently no one region is continuously in contact with the bottom. During the inhibition period, however, and for the first day or two after return to water there is little or no motor activity and one side of the piece may be continuously in contact with the bottom. The effectiveness of contact and nearness to the bottom in determining basal ends has been shown in many other experiments (Child, '26*b*, '27*a, b*). In the cases under consideration the inhibiting effect of contact and that of the inhibiting agent are additive and the position of the new basal region one one side of the stem may be a resultant of the two factors or the effect of either alone. The important point is that inhibition of the middle region of the bipolar form, whether by chemical agents or by contact with the bottom or by the combined action of both may determine a basal region there.

Torrey ('10) found that the development of a basal region from a lateral stem region might be determined by the more distal levels of the axes already present and without the action of external factors, in other words, under the dominance of the higher levels of the gradients. In the experiments with inhibiting agents, however, the dominance of the more distal levels is undoubtedly decreased, consequently it cannot be a significant factor in determining the basal region in these cases. This dominance does serve to determine the middle region as the lowest physiological level of the bipolar form, but the inhibiting action of the agent or of contact or of the two combined is required to lower it still further to the level at which the development of a basal end becomes possible.

The more proximal levels and the basal end in these bipolar-unipolar transformations usually attain finally a relatively larger size than under normal conditions. Under all inhibiting conditions used a similar change in proportion from the normal has been observed. This is a result of the differential susceptibility at different levels of the axis. The most active apical regions are more inhibited than the less active proximal and basal regions, consequently the latter are able to obtain a proportionally larger share of the available nutrition or even to grow at the expense of more distal levels and so to become relatively larger than in the normal animals.

The probable transformation of bipolar into unipolar forms by obliteration of the two original polarities and determination of a single new polarity is also easily accounted for in terms of the gradient. Obliteration of the original polarities by the inhibiting agents is a consequence of differential susceptibility of different levels of the gradient. The higher levels being more susceptible are more inhibited than the lower and with certain degrees of inhibition the normal differences of different levels may be greatly decreased or completely obliterated, so far as any appreciable effect is concerned. In the cases of transformation this obliteration is accompanied by regression, involving complete disappearance of the more specialized parts. Under these conditions the differential of position may be effective in determining a new polarity, the upper, freely exposed portion becoming apical, the region in contact or near the bottom basal, as in other cases of obliteration of old and determination of a new polarity by the differential exposure (Child, '27*a*).

The development of unipolar from bipolar forms by the death of one axis and persistence of the other is not, strictly speaking, an axial transformation, but merely persistence of an axis. The inhibiting conditions kill one axis and not the other because one is more susceptible than the other. In cases of this sort in which it has been possible to distinguish distal and proximal axes of a bipolar form, the distal axis has been found the more susceptible, as might be expected from its development at a higher level of the original gradient of the stem than the proximal. This difference in physiological condition of the two axes is of interest as indicating that even though one of them, the proximal, represents a reversal of the original polarity it still retains traces of its origin from a lower gradient level than the distal axis. Such difference in susceptibility of the two axes is usually found in the longer bipolar forms in which the difference in level of stem at which the two polarities develop is considerable. In the shorter forms with little or no stem between the two hydranths (Fig. 3) there may be no appreciable difference in susceptibility between the two hydranths and both axes may undergo regression equally (Fig. 19).

Persistence of one of the two axes evidently means that some difference in condition persists in the original direction of the gradient. The reorganization which usually occurs with development of a hydranth much smaller than the original and of a basal region consists in development of stem and basal end from what was in some cases originally the proximal part of the hydranth. Apparently the higher levels of the gradient are lowered by the inhibiting conditions more than the lower levels so that apical regions are relatively smaller, proximal and basal regions relatively larger than under normal conditions. This again is a consequence of the differential susceptibility of the different levels of the gradient.

The interpretation of the experimental data in terms of gradients is simple and is in complete agreement with the results of various other lines of experimentation both on *Corymorpha* and on other forms. The existence of the gradients has been demonstrated by many different methods and the differential susceptibility of different levels has been proved beyond doubt, both by differences in survival time and by differential modification of development with many different agents. No other interpretation has as broad an experimental basis as this.

SUMMARY.

1. Bipolar forms resulting from reconstitution of stem pieces of *Corymorpha palma* can be transformed into bipolar-unipolar forms by subjection to inhibiting conditions for various lengths of time. The transformation consists of development of a basal end from the lateral stem region between the hydranths.

2. The development of the base may result from the action of the inhibiting agent in lowering the physiological level of the region concerned to the level at which base development is initiated and the more intimate and continuous contact with the bottom in consequence of decreased motor activity under inhibiting conditions may act in the same direction and contribute to the result.

3. Bipolar forms may become unipolar by the death of the parts representing one axis because of higher susceptibility. In such cases extensive reorganization along the persisting axis occurs.

4. It is believed that inhibiting conditions may bring about transformation of bipolar into unipolar forms by obliteration of the two original polarities through differential susceptibility and the determination of a single new polarity by the differential of position.

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PHYSIOLOGICAL POLARITY AND DOMINANCE IN THE HOLDFAST SYSTEM OF *CORYMORPHA.*

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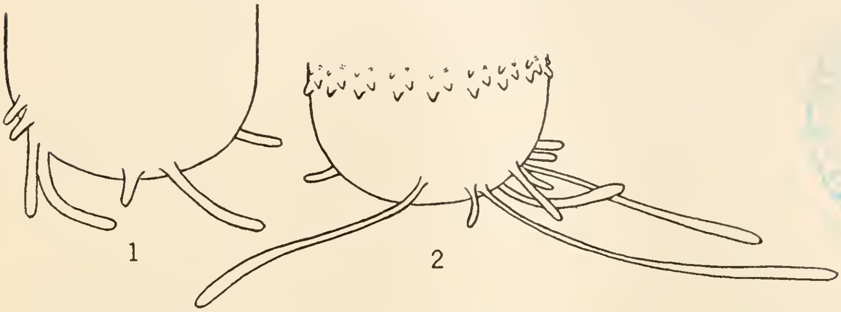
Corymorpha palma is found on tidal flats and anchors itself on the substratum of soft mud or fine sand by means of a large number of filamentous, stolon-like structures covered with a delicate perisarc, which extend from the basal region of the hydroid and serve as holdfasts. Torrey ('04, '07, '10*a*, '10*b*) has described the structure, the embryonic and reconstitutional development and some of the reactions of these holdfasts, viz., their extremely rapid elongation, the amœboid activity of their tips, their positive geotropic reaction and the ability of actively developing holdfasts to inhibit the development of others distal to them.

The holdfasts are much more slender than the usual hydroid stolon, being only about 0.1 mm. in diameter at the tip which is the region of greatest diameter except in early stages, but they are similar to other hydroid stolons in appearance and development. Apparently, however, they are somewhat more specialized than the ordinary stolon for, so far as known, they do not transform into, or give rise to hydranth-stem axes when isolated from the parent stem, but always remain holdfasts. If they really lack the capacity to give rise to hydranth-stem axes they should, strictly speaking, be designated as holdfasts rather than as stolons. Nevertheless it seems evident that they represent merely a specialized form of stolonic axis. Each holdfast evidently represents a physiological axis and possesses a definite polarity which appears in its form, the manner of its development and its final exhaustion. Moreover, the holdfast system of the individual hydroid shows certain definite relations to the polarity of the whole, both in its origin and in the relations of its members. The present paper is primarily concerned with those aspects of holdfast life which are related to physiological polarity, viz., the manifestations of polarity in the individual holdfast and the relations of the holdfast system to the polarity of the whole animal.

THE INDIVIDUAL HOLDFAST AS A PHYSIOLOGICAL AXIS.

The Gradient of the Holdfast.—The holdfast originates as a bud in the basal region of the hydroid and develops as an axis with a growing tip. Like other buds, it arises as a localized region of cellular activity which decreases in intensity from a central region peripherally. As the holdfast develops the most active region necessarily becomes the tip and the radial gradient in activity which constitutes the first step in the development becomes an axial gradient with its high end at the tip. The existence of this gradient from the earliest localization of the holdfast bud has been demonstrated by its differential susceptibility to various agents, by differential vital staining, by differential reduction of vital dyes and $KMnO_4$ and by the differential in the indophenol reaction. All the methods used agree in showing that the tip of the holdfast is the high end of the gradient. The results obtained on the animal as a whole with these various methods have been described in an earlier paper (Child, '26*a*) and since all methods agree as regards the holdfast, it seems unnecessary to give the data in detail. Moreover, the gradient is obvious in the general behavior of the holdfast. It may be noted, however, that the various agents penetrate the very thin perisarc almost at once and with those agents which afford a means of following the penetration directly by staining it is found that the older perisarc farther from the holdfast tip is usually penetrated before that at the tip. Evidently the very conspicuous gradient of the holdfast cannot be ascribed to differences in permeability of the perisarc.

The holdfast bud or primordium may persist for a long time without appreciable growth beneath the perisarc of the stem, but remains capable of very rapid outgrowth when conditions permit. As a bud it usually tapers slightly toward the tip, but when outgrowth begins its diameter is for a time about the same throughout its length. With further outgrowth and particularly after it comes into contact with the substratum, the tip retains its diameter, or the diameter may appear to increase somewhat because of flattening of the tip on the substratum, but proximal to the tip the diameter gradually decreases until the proximal portions of the longer holdfasts are extremely slender threads. This decrease in diameter is the result of gradual atrophy of the cells in the more proximal regions of the elongating holdfast until there is nothing left except the tube of very delicate perisarc secreted by the holdfast as it elongates and within it traces of cellular debris. Figs. 1 and 2 show various stages in the development of holdfasts. In these and all following figures perisarc is indicated by a heavy line.



Figs. 1 and 2. Activation and outgrowth of holdfasts. Fig. 1, proximal cut end of stem piece 1 1/2 hours after section, showing activation and outgrowth of holdfasts from very early bud stages present before section. Fig. 2, a proximal cut end 24 hours after section, showing a stage of development of a second set of holdfasts 3 hours after removal of first set. A new zone of holdfast buds has begun to develop a short distance distal to the active holdfasts.

Atrophy of the more proximal levels is characteristic of the true stolons of other hydroids (Child, '23) as well as of these holdfasts, another fact which indicates the physiological similarity of true stolon and holdfast. It was pointed out in the paper just referred to that this atrophy is clearly a consequence of the gradient in the stolon. While the outgrowth is short all parts obtain nutrition from the hydroid body, but it appears that after a certain length is attained this is no longer possible and from this stage on the outgrowth is gradually undergoing starvation and reduction. Very generally, if not always, in the starvation of the simpler animals the parts which are most continuously and most intensely active decrease in size less rapidly than the less active because they are able to live and more nearly maintain themselves at the expense of the less active. Microscopic examination of the cells of the longer holdfasts shows that those at or near the tip appear to be in good condition while evidences of inanition increase with increasing distance from the tip. Toward the proximal end the cells become more transparent and the holdfast has decreased in diameter more than the perisarc so that only strands of protoplasm here and there are in contact with the perisarc. Finally at a greater or less distance from the tip nothing remains within the perisarc but a few granules. The transition from the region of good condition at the tip to that of complete atrophy is usually very gradual, as indicated in Fig. 3, but occasionally it is rather abrupt, particularly in the earlier stages of the atrophy (Fig. 4). The methods used to demonstrate the gradient indicate that such differences are due to individual differences in length and slope of the gradient in the holdfast and such differences are undoubtedly associated with differences in physiological condition and rate of elongation in the different holdfasts.

The holdfast, like the stolon of other hydroids is obviously a gradient in which the characteristic process, at least in the earlier stages, is continued growth of the tip. As a growth gradient it is also similar to rhizoids, roots and various forms of hairs in plants and differs from the chief plant axis only in its inability to give rise to buds.

The Motor Activity of the Holdfast.—Holdfasts growing rapidly from the cut end of a stem as in Figs. 1 and 2 often show slight motor activity before they come into contact with the substratum. Their movement consists in bending in various directions and in some cases slight contraction and extension are observed. All such movements are slow, much slower than tentacle movements. Motor response of holdfasts to direct external stimulation has not been tested.

As soon as the holdfast comes into contact with the substratum it adheres and further motor activity is limited to the tip. In nature the holdfasts burrow through the mud, but in glass containers in the laboratory elongation after contact takes place along the surface of the substratum. (Torrey, '10*b*) has noted that the elongating holdfast is positively geotropic. After attachment, however, the holdfast tip often shows more or less amœboid activity, as Torrey ('04, p. 416) has also noted. Elongated pseudopodia have not been observed, but the distal region may undergo change in shape, the activity decreasing from the tip proximally. Fig. 5 shows a series of outline drawings of a holdfast tip at intervals of one to two minutes and serves to indicate the character and range of amœboid activity observed. In general this activity has seemed to be greater in holdfast tips farther away from the stem, rather than in the earlier stages of outgrowth, but it cannot be positively asserted that this is the general rule. Occasionally the extreme tip has been seen to undergo slight retraction, 0.03–0.07 mm., followed after a minute or two by renewed advance. So far as observed, the amœboid activity occurs chiefly at the extreme tip and decreases rapidly in the proximal direction and at a distance of 0.3–0.5 mm. from the tip is no longer visible.

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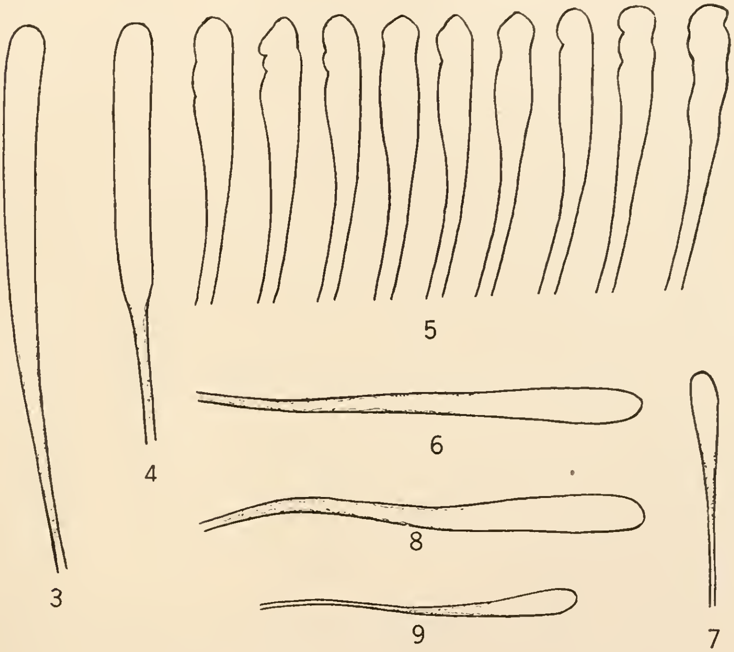


Figs. 3–9. Individual holdfast tips. The shrunken appearance of the proximal cœnosarcal regions is indicated by dotted lines inside the heavy lines indicating perisarc. Fig. 3, a holdfast which has covered a distance of 7–8 mm., showing gradual decrease in diameter and increase in degree of atrophy in proximal direction. Fig. 4, a holdfast which has progressed 7–8 mm., showing rather abrupt decrease in diameter and increase in degree of atrophy. Fig. 5, a series of outlines of a holdfast tip sketched at intervals of 1–2 minutes to show the amœboid change in form. Fig. 6, a holdfast which has covered 15 mm. Fig. 7, a holdfast which has covered 20 mm. and is approaching exhaustion. Fig. 8, an earlier, and Fig. 9 a later stage of a completely isolated holdfast.

The Later Stages of Holdfast Development.—When the holdfast attains a certain length which varies in different holdfasts, the atrophy of the more proximal regions brings about complete separation of the distal portion of the cœnosarc from the hydroid stem. The begining of this atrophy usually becomes evident in the decrease in diameter of the proximal region when the holdfast has attained a length of 2–3 mm. (Fig. 2) and by the time it has reached a length of 4–8 mm. the living cœnosarc of its distal end is apparently completely separated from the parent stem, the perisarcal tube being the only connection (see Figs. 12, 13).

Advance of the tip does not cease when this isolation occurs. The more proximal portions evidently serve as nutrition for the distal parts and the progress of the tip with the secretion of perisarc continues with gradual decrease in length and diameter of the cœnosarc until finally exhaustion occurs. Figs. 3, 4, 6, 7 illustrate the decrease in size of the tip. Figs. 3 and 4 represent the distal portions of holdfasts which have grown a distance of 7–8 mm. from the stem, Fig. 6 is a distal end 14–15 mm. and Fig. 7 another 20 mm. from the stem and almost exhausted. Advance does not continue much beyond the stage of Fig. 7. Apparently the gradient differences at this stage are no longer great enough in the small fraction of the original gradient which remains so that the tip can maintain itself and continue to advance at the expense of lower levels, *i.e.*, the degree of starvation has become almost as great at the tip as elsewhere. After its advance ceases the tip may continue to live and decrease in size still further for at least a week, perhaps more.

That the continued advance of the tip is entirely independent of connection with the parent hydroid is evident from the fact that it is not at all affected by physical isolation of the tip from the hydroid. Fig. 8 represents a holdfast from which the perisarcal tube connecting it with the parent stem has been cut away. This tip continued to advance, secreting the perisarcal tube exactly as if connected with the stem. Fig. 9 shows a later stage, approaching exhaustion. When a stem is removed after giving rise to holdfasts the latter remain attached to the substratum and continue to advance until they are exhausted.

In this continued advance of the tip after complete isolation from the hydroid the holdfast also resembles the true stolon of other hydroids (Child, '23). In both the advance continues long after physiological connection with the hydroid stem is lost and until the cœnosarc is reduced to a small fraction of its original size. Even when the advance ceases the cells of the tip are visibly in better condition than those of lower levels.

In the laboratory holdfasts have been observed to advance over a distance of 2–3 cm. before exhaustion and the binding of the mud about the base of the hydroid in nature indicates that under natural conditions they may cover a somewhat greater distance than this.

Torrey ('04, p. 416, '07, pp. 277–8) regards advance of the holdfast tip as due primarily to amœboid activity at the tip which stretches the holdfast. He states, however, that when stems are suspended free in water the holdfasts extend in all directions. On cut stems also the holdfasts elongate very rapidly before they come into contact with the substratum at all (Figs. 1 and 2). Such elongation when the tip is not in contact certainly cannot be due to amœboid activity for the tip cannot exert tension. Moreover, the volume of the holdfast cœnosarc increases very rapidly and very greatly during the earlier stages. And finally, the progress of the tip is not necessarily in a straight line, but the direction may undergo frequent change, even on smooth glass so that the perisarcal tube is sinuous or forms a circle or a spiral (see Figs. 12 and 13). If any actual stretching occurs it must take place only very near the tip and must very soon reach a limit for the perisarc is not torn away from the substratum and straightened and the cœnosarc is not left behind. It seems certain that at least the earlier stages of holdfast elongation are not due merely to the amœboid activity of the tip. To all appearances extensive growth occurs, although it is conceivable that cells migrate from the parent stem into the holdfast. Evidence of such migration, however, is lacking.

To what extent the later advance of the holdfast tip after isolation of its cœnosarc from that of the parent stem may be due to amœboid activity and to what extent to actual growth of the tip at the expense of more proximal levels is less readily determined. The appearance of the cells at the tip suggests continued growth, but the very rapid advance suggests amœboid activity. The perisarc is certainly continuously secreted as the tip advances, rather than elongated by stretching. The planula of *Corymorpha* often progresses for some distance over the substratum leaving a delicate perisarcal tube behind it as it travels (Child, '26*a*). In that case the advance is evidently due to motor activity rather than growth. It seems improbable, however, that the holdfast changes completely its activity from growth to amœboid activity after it comes into contact with the substratum. Amœboid activity has been observed in tips which are approaching exhaustion and have almost ceased to advance. In the light of all the facts it seems probable that more or less growth of the tip at the expense of proximal levels does occur even after isolation from the stem, although amœboid activity may play a part in the advance of the tip in later stages.

The continued advance of the tip until exhaustion occurs and its maintenance in good condition at the expense of more proximal regions with progressive atrophy of the proximal parts and the amœboid activity of the tip are evidently all expressions of the physiological gradient characteristic of the holdfast. In fact, it does not seem possible to account for its behavior except in terms of a gradient. Moreover, the gradient accounts adequately and entirely for all the phenomena of polarity which the holdfast exhibits.

The Rate of Advance of the Holdfast.—The advance of the holdfast is so rapid that with a low magnification and an ocular micrometer the advance from minute to minute can be directly observed. Different holdfasts show different rates of advance, but in general the rate decreases very greatly in the later stages and advance finally ceases. The following table gives a few characteristic measurements of the rate of advance. Nos. I., II. and III. are the three longest holdfasts of Fig. 2 measured at a stage a few minutes later than that represented in the figure, *i.e.,* about three hours after section of the stem and activation of the holdfasts. These early stages were elongating at the rate of a millimeter in seventeen to twenty-two and one half minutes, the highest rates observed. Nos. IV., V. and VI. are holdfasts twenty-four hours old, growing from an intact base after removal of the original holdfasts. In these the rates of advance are much slower

TABLE I.

RATES OF ADVANCE OF HOLDFAST TIPS.

		Distance of Tip from Parent Stem in Mm.	Time in Minutes and Seconds to Cover 10 Micrometer Divisions (0.33 mm.)
Holdfasts from cut end of stem 3 hrs. after section	I	3	7:30
	II	4	6:40
	III	5	5:40
Holdfasts 24 hrs. old from intact basal end after removal of original holdfasts	IV	8	14
	V	15	20
	VI	20	46

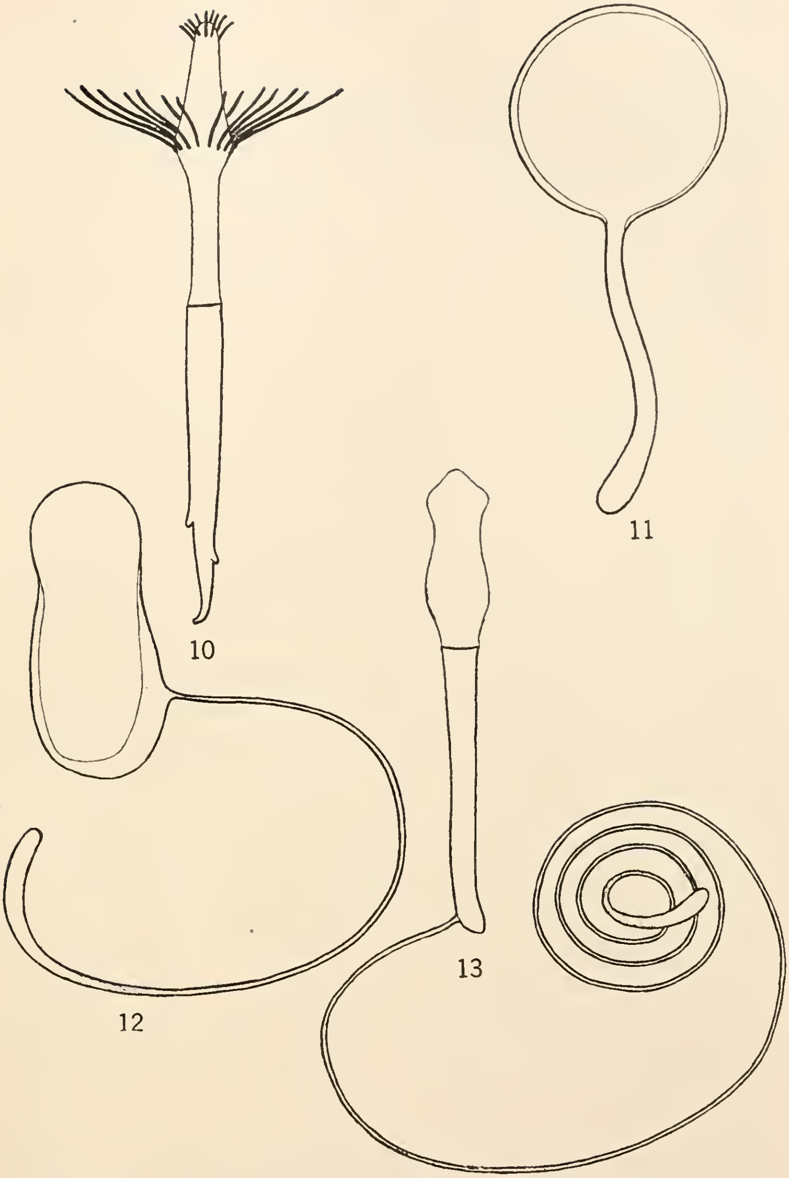
and it will be observed that they decrease to a marked extent with increasing length of the holdfast. These three cases are the three holdfasts of Figs. 4, 6 and 7 at the stages figured. Fig. 7, No. VI. in the table, was approaching exhaustion at the time of measurement and its rate of advance is only about one eighth that of No. I. A number of other measurements made all fall within the extremes of the table. Torrey ('04) mentions a rate of nine micra per minute in one case; this would equal 0.33 mm. in more than thirty-three minutes, a rate much slower than those of the earlier stages in the table. Elsewhere (Torrey, '07) he mentions a rate of 15 mm. per day which is almost midway between the rates of Nos. V. and VI. of the table. The rates in Table I. are all from holdfasts in contact with the substratum. The rates of the earlier stages seem high for a growth process, but rates approaching these are found in holdfasts which are not yet in contact with the substratum. Fig. 1, for example, shows a basal cut end 1 1/2 hours after the cut was made. Before the section only very early holdfast primordia were present but within the short time following section some holdfasts attained a length of 1 mm. before their tips came into contact with the substratum. Most of the length of 1 mm. was covered during the last half hour of the period. Except for possible migration of cells from the stem into the holdfast this elongation appears to be due to growth and the rate of elongation is nearly as high as in some of the holdfasts after contact. The holdfast appears to be primarily a growth gradient with amœboid activity at its tip.

Dominance and Physiological Isolation in the Origin and Development of the Holdfast System.

In later stages of the hydroid development the holdfast buds arise in regular order along the entodermal canals in the basal region and show in their origin and development certain definite relations to the polarity of the whole animal and to each other.

Early Normal Development.—The first holdfasts arise as minute buds at or near the basal end of the perisarcal region of the young hydroid, but they appear only after the young hydroid has become attached by its perisarc and they remain as minute buds under the perisarc for some time after their appearance. Under good conditions the first holdfasts appear only when the hydroid has attained the stage shown in Fig. 10 and often not until still later. In general holdfast development begins at or near the basal end and progresses distally from it, but the early holdfasts are more or less irregularly scattered in position. In vigorous, actively growing young animals the holdfast buds do not grow out through the perisarc of the stem at once after their formation, but develop very slowly for a time, remaining buds and not beginning active outgrowth until the animal attains a considerable length. During this earlier stage they are merely primordia of holdfasts and play no part in attaching the animal unless they happen to be terminal. This very slow development until the animal attains a certain length suggests that the holdfast buds, like the stolons of other hydroids are inhibited in development by apical regions until the increase in length of body brings about a certain degree of physiological isolation.

Premature Development of Holdfasts under Inhibiting Conditions.—Under conditions which inhibit development to a slight extent holdfasts may not only appear as buds, but may actually grow out and attach themselves at much earlier stages of development than those at which they normally appear. In low concentrations of KCN, alcohol, ether, etc. and even when kept in sea water several centimeters deep without change or aeration the



Figs. 10–13. Development of holdfasts in young animals. Fig. 10, young hydroid in stage at which holdfast buds usually first appear under normal conditions. Fig. 11, outgrowth of a holdfast from a blastula under slightly inhibiting conditions. Fig. 12, a planula with holdfast which appeared in blastula stage under inhibiting conditions. Fig. 13, slightly inhibited young hydroid with long holdfast which appeared in the early planula stage. Hydroid and holdfast are figured in the same plane, though in nature the plane of holdfast elongation is at right angles to the hydroid axis.

holdfasts develop in the planula or even in the blastula stage. Fig. 11 shows a blastula with holdfast. Fig. 12 a planula with holdfast which began its development in the blastula stage, Fig. 13 an early hydroid with holdfast which appeared in the early planula stage twenty-four hours before the stage of the figure. The spiral growth seen in Fig. 13 is of frequent occurrence in the holdfasts, but the conditions which determine it are unknown. These three, as well as numerous other similar cases observed are from material developing at the bottom of 5 cm. depth of standing water without aeration or change since early cleavage stages. As compared with development in frequently changed, well aerated water, this material was distinctly inhibited in development. Many similar cases of premature development of holdfasts have been observed with low concentrations of various inhibiting agents. In fact, the early formation and outgrowth of holdfasts is the characteristic effect of a slight degree of inhibition or retardation of development.

In this connection it may be recalled that development of stolons instead of the hydranth-stem axis from the planula has been experimentally determined in a campanularian hydroid, *Phialidium gregarium,* by various inhibiting conditions (Child, '25). In that form both ends of the planula may give rise to stolons. In *Corymorpha* the premature development of holdfasts has been observed only from the basal region and the hydranth-stem axis has developed sooner or later, but always retarded. It has been pointed out elsewhere that the polarity of the tubularian hydroids is apparently less readily altered than that of the campanularian (Child, '19, '25, '26*a*) and the persistence of the planula polarity in *Corymorpha* under inhibiting conditions constitutes further evidence along the same line.

The much earlier appearance and outgrowth of the holdfasts under slightly inhibiting than under normal conditions shows that the inhibiting conditions favor in some way their development. But it is evident that the inhibiting conditions are not in themselves necessary for development of holdfasts, since in larger older animals the holdfasts develop very rapidly in the best possible laboratory environment (Figs. 1, 2). As regards the manner in which the inhibiting conditions induce premature development of holdfasts, two possible factors suggest themselves. First, the inhibiting conditions may directly affect the lower end of the primary gradient which is to become the basal region and lower its metabolic or physiological level so that conditions favorable to holdfast development arise earlier than they would under normal conditions, in which they appear only after the animal has become considerably older and its metabolism has undergone decrease. It has been shown elsewhere (Child, '27*a*, *b*) that basal regions may be determined and localized by local inhibiting conditions and it is possible that the direct effect of the inhibiting conditions in decreasing the activity of the lower end of the gradient may be concerned in determining holdfasts at an earlier stage of development than that at which they normally appear.

A second possible factor in the premature development of holdfasts under inhibiting conditions is an increase in the degree of physiological isolation of the lower end of the gradient from apical dominance in consequence of the effect of the inhibiting conditions on the apical region. The fact that the holdfasts are not only prematurely determined as buds but also develop prematurely with great rapidity indicates that the effect of the inhibiting conditions on their development is, at least in large part, indirect rather than direct, *i.e.*, an increase in the degree of physiological isolation of the basal region from apical dominance resulting from the inhibiting action of the conditions on the apical region. Under normal conditions such physiological isolation does not occur until a much later developmental stage when the length of the hydroid begins to exceed the range of that degree of dominance necessary for complete inhibition of holdfast development, and even then their development is very slow until a considerably greater length of stem is attained, which apparently permits a greater degree of physiological isolation. The dominant apical region is more susceptible than the less active basal region to the inhibiting conditions (Child, '26*a*) and its dominance over the basal region is decreased, consequently holdfasts are able not only to appear as buds, but to grow out at once. The holdfast evidently represents a new physiological axis which can be formed in the basal region of the hydroid only when a certain degree of physiological isolation from the dominance of the apical region occurs. A similar relation between hydranth region and stolon exists in *Tubularia* (Child, '15, pp. 91, 92, Figs. 42, 43) as well as in other hydroids, but in those forms the stolon tip transforms into a hydranth-stem axis as soon as the degree of isolation is sufficient.

This interpretation of holdfast development does not conflict in any way with the fact that new basal ends on which holdfast buds may develop and grow out are often determined in pieces undergoing reconstitution by the inhibiting conditions associated with contact or nearness to the bottom (Child, 26*b*), especially with the addition of inhibiting agents (Child, '27*a*). In normal development and in many cases of reconstitution the basal region is apparently largely or wholly determined by internal factors as a secondary gradient at the low end of the primary gradient (Child, '26*b*). Apparently it represents a region sufficiently isolated physiologically from apical dominance to develop a new gradient opposite in direction to the old and to grow in length, but still to some extent under apical dominance since it possesses the capacity to develop a hydranth-stem axis from its proximal end (Child, '26*b*, p. 786), but does not do so except after separation from the apical and inhibition of its own distal region. The late appearance and slow development of holdfasts in normal young animals suggests that a further increase in the degree of physiological isolation of the basal region is necessary for holdfast formation. Since a condition of relatively low metabolism is evidently the essential factor in determining a basal region, local external inhibiting conditions may determine such a region or assist in determining it, by establishing the proper metabolic conditions. Whether or when holdfasts shall develop on a basal region thus determined apparently depends on the degree of physiological isolation from the dominant apical region which is attained in such a region. In the piece undergoing reconstitution the new apical region is often not sufficiently developed at the time the basal region is determined to prevent the appearance of holdfast buds; but apparently it often does inhibit their further outgrowth for usually they develop very slowly until the animal attains a certain length and in the very small individuals from short pieces they may remain buds indefinitely. In both reconstitution and embryonic development, however, inhibition of hydranth development favors their early outgrowth unless the inhibiting conditions are sufficient to inhibit the holdfast buds directly.

Physiological Dominance and Isolation among the Holdfasts.—As noted above, the earlier holdfasts are scattered about the basal end, but after the longitudinal entodermal canals develop, the later holdfast buds arise in regular order along these canals, usually two rows to a canal, forming a definite zone surrounding the stem several millimeters from the basal tip. In this zone the first buds to appear and the most advanced in development at any given time are those nearest the basal tip and bud development progresses distally. Fig. 14 shows the buds on a single canal as they appear in the older animals and in Fig. 2 a new zone of buds is developing distal to a cut end.

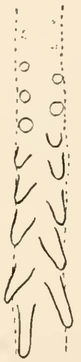


Fig. 14. The system of holdfast buds of a single entodermal canal as it appears in older animals.

This definite order of development and spatial arrangement also suggests the existence of a physiological relation between the individual holdfasts of the system and Torrey has observed that the removal of the older holdfasts accelerates the development of those near the wound on the distal side (Torrey, '10*a*, p. 217). In the normal development of the animal the holdfasts nearest the basal tip grow out first when the animal attains a certain length. After protoplasmic connection between their tips and the stem is severed by atrophy of the connecting region resulting from elongation the buds next in order distally grow out and so on. Meanwhile new buds may begin to develop at the distal end of the bud zone. This zone therefore gradually changes its position in the distal direction and in the larger individuals is often entirely above the surface of the mud in which the basal end is buried. All these facts indicate the existence of a relation of dominance and subordination in the holdfast system, the holdfasts at the proximal end dominating those distal to them. In this relation the holdfasts which develop from the higher levels of the basal gradient dominate those of lower levels, just as in other similar gradients, *e.g.*, many plant axes.

After the zone of holdfast buds has developed, the buds of any level of this zone can be activated at once by section of the stem just proximal to them. Such section removes the proximal dominant buds or holdfasts and the most proximal buds remaining grow out and become dominant. This activation and outgrowth take place very rapidly. Fig. 1, for example, shows the proximal end of a stem 1 1/2 hours after section through the distal region of the holdfast zone. Before section the holdfast buds in this region were in very early stages like those of the upper third of Fig. 14. Within the short time of 1 1/2 hours some of these buds attained a length of almost 1 mm.

Fig. 2 shows a stem in which a second set of holdfasts is developing after removal of the first set which developed after removal of the basal end 24 hours preceding the stage figured. The first set of holdfasts was allowed to develop for 21 hours at which time those which developed first had lost cœnosarcal connection with the stem and others distal to them were developing. Then all developed holdfasts were removed. Fig. 2 shows the development attained by the new set three hours after removal of the old. Fig. 2 also shows a new zone of holdfast buds developing at a short distance from the proximal end. This zone has developed within 24 hours since it was not present at the time of section. Such a new bud zone has been observed in other similar cases and in all, as indicated in Fig. 2, it arises a short distance distal to the actively growing holdfasts, not immediately adjoining them. This position is characteristic and again indicates that these rapidly growing holdfasts dominate a certain length of stem in such a manner as to prevent development even of other holdfast buds within that region. The following experimental data show that the rapid reaction following section of stem or removal of holdfasts in the cases above described is not exceptional.

I. Twenty stem pieces comprising the proximal fifth of the naked region and the distal half of the perisarcal region were cut from newly collected, relatively young individuals 25–30 mm. in length. These pieces were approximately one fourth the total stem length. They possess only very early holdfast buds or no visible buds. After 1 1/2 hours three pieces were attached by their holdfasts, some of which were 1 mm. long (Fig. 1) and eight others showed outgrowing holdfasts at the proximal end. After 14 hours nineteen pieces were attached; of these fifteen showed a maze of holdfasts about their bases, some of them several mm. in length, and four pieces were attached by the perisarc without holdfast outgrowth.

II. Six pieces from the preceding experiment were detached and all developed holdfasts were removed from the proximal end after 21 hours growth. One hour later two cases showed outgrowing holdfasts, one a single holdfast 0.23 mm., the other, two, each 0.17 mm., both attached to the bottom. After 1 1/2 hours four pieces showed outgrowing holdfasts and three were attached by them. After 2 hours all six showed growing holdfasts and four were attached. One holdfast was 1.3 mm. in length. After 3 hours four were attached. Fig. 2 represents one of the pieces at this stage. After 4 hours, five, and after 9 hours, all were attached by holdfasts. Five of the six pieces developed a second zone of holdfasts buds like Fig. 2 within 33 hours or less.

III. From ten newly collected individuals 50–60 mm. in length hydranths and basal ends, including all visible holdfast buds, were removed. The earlier stages of new holdfast development were not observed, but after 18 hours eight were attached with numerous holdfasts some 3–4 mm. in length. Two pieces were still unattached, but show early developing holdfast buds. In this experiment preformed buds were absent but the formation and development of new holdfasts to a length of 3–4 mm. has occurred within 18 hours.

IV. Ten stems similar to III. After 22 hours five were attached and some holdfasts were 3–4 mm. long. All five showed a new zone of holdfasts developing 1–2 mm. distal to the outgrowing holdfasts. Five were unattached, one with early holdfasts, four without holdfasts. After 46 hours all had developed holdfasts and become attached.

V. In this experiment four larger animals, 60–70 mm. in length, which had been 6 days in the laboratory, were used. In these the holdfast bud zone had already developed above the level of the mud, see p. 30). These were the largest individuals obtainable during the summer of 1926, when most of the observations on holdfasts were made. The basal ends were removed at such level as to leave 6–8 rows of preformed holdfast buds distal to the cut end, and the hydranths were also removed. After 2 hours the most proximal one or two rows of buds were elongating and after 8 hours all four pieces showed holdfasts 0.5–0.8 mm. in length and two were attached by their holdfasts. The much slower outgrowth in this, as compared with other experiments is doubtless due to the six day period in the laboratory without food preceding the experiment.

These few experiments are sufficient to indicate the extremely rapid activation of preformed holdfast buds and development of new buds and holdfasts after removal of levels proximal to them.

Conclusion.

The *Corymorpha* holdfast is a tertiary axis which develops from the secondary axis which constitutes the basal region. It resembles the stolons of other hydroids in appearance and in its growth and development, but is apparently more specialized than the true stolon, since it is not known to give rise to a new hydranth-stem axis as the stolon does when isolated. All experimental methods applied to the holdfast indicate that it represents a physiological gradient with high end at the tip, like a hydroid stolon, a plant root or rhizoid and many other physiological axes. Moreover, the developmental behavior of the holdfast, the greater amœboid activity of its tip as compared with other parts, the maintenance in good condition of distal, at the expense of proximal regions, even during gradual reduction by starvation, all constitute further evidence that it is a gradient. And there is absolutely nothing to indicate, and no ground for assuming that its axiate character has any other basis than this gradient.

The secondary gradient which characterizes the basal region apparently represents a certain degree of physiological isolation from apical dominance and the slow appearance and development of the holdfasts under normal conditions, as compared with their premature development under inhibiting conditions suggest that a still higher degree of physiological isolation is necessary for their development than for the determination of the basal end. Since the apical region is more susceptible to inhibiting conditions than the basal (Child, '26*a*), these conditions decrease apical dominance without greatly affecting the holdfasts themselves, *i.e.*, they inhibit the internal factors which inhibit or retard the outgrowth of the holdfasts and so increase the degree of physiological isolation in the holdfast region.

When active development of the holdfasts does begin, a relation of dominance and subordination arises within the holdfast system. The most proximal holdfasts, *i.e.*, those nearest the basal tip, are the first to appear, as might be expected, since the degree of physiological isolation must be greater there than in more distal regions. These first holdfasts dominate buds distal to them and prevent or retard their development. Removal or outgrowth and separation of these most proximal holdfasts permits the further development of those next distally and so on. This relation is essentially identical with that found in many axiate complexes of plants.

Concerning the nature of this dominance in *Corymorpha* nothing definite can be said. The very wide occurrence in both plants and animals of essentially identical phenomena of dominance, subordination and physiological isolation and the apparently primary dependence of dominance on high metabolic activity, rather than on any particular kind of activity indicate the non-specific character of dominance and suggest that it is primarily dynamic rather than a matter of specific chemical substances.

The holdfasts are also of considerable interest because of their extremely rapid activation and development after the bud stage. The outgrowth of holdfasts within an hour after removal of more proximal regions represents an unusually rapid reaction and the advance of the tip in later stages, even after isolation from the stem may be as rapid as, or even more rapid than the early elongation. The question whether elongation of the holdfast is due to actual growth, or, as Torrey ('04, '07) believes, to the amœboid activity of the tip, or as to the relative importance of these two factors, cannot be finally answered at present, but it seems evident that the earlier elongation of the holdfast, which may take place free in the water without contact of the tip cannot be due to the amœboid activity of the tip. Migration of cells from the stem into the holdfast is possible, but there is no evidence that it occurs. To all appearances this elongation is due to real growth. As regards the later advance of the tip after isolation and while it is undergoing reduction and atrophy of the more proximal regions is occurring, only further investigation can determine to what extent growth of the tip at the expense of other parts and to what extent amœboid activity is concerned. But whatever the processes underlying particular aspects of its behavior, the holdfast originates as a local region of some sort of physiological activity which decreases peripherally from a center. This activity leads to outgrowth and elongation and the central, most active region necessarily becomes the tip of the outgrowth and the radial gradient becomes an axial gradient. All the behavior of the holdfast is an expression of that fact.

Summary.

1. The holdfasts of *Corymorpha* are tertiary axial gradients developing as buds from the secondary gradient of the basal region.

2. Their premature appearance and outgrowth in embryonic development under inhibiting conditions, as compared with their much later appearance and slower growth in development under normal conditions, suggest that a certain degree of physiological isolation from apical dominance is necessary for their development.

3. As the holdfast elongates, the cœnosarc of its proximal region atrophies so that the distal portion becomes completely isolated from the parent stem, but advance of the tip continues until exhaustion occurs, the distal cœnosarc remaining in good condition while atrophy continues at the proximal end. The delicate perisarcal tube secreted as the tip advances is the only connection with the stem in later stages.

4. Holdfast elongation is extremely rapid, sometimes 1 mm. in 15–20 minutes. After a few hours the rate of elongation decreases and exhaustion occurs after 2–3 days. The maximum lengths observed are 2–3 cm.

5. The holdfast tip in contact shows some amœboid activity and while the earlier stages of elongation are apparently due largely or wholly to growth and may occur with the tip free in the water, the continued advance over the substratum after isolation from the stem and while undergoing decrease in size may be due in part to the amœboid activity of the tip.

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MORPHOLOGY AND BINARY FISSION OF *PERANEMA TRICHOPHORUM* (EHRBG.) STEIN.

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Introduction.

In view of the somewhat unsatisfactory classification of the colorless euglenoids in use prior to 1925, it has seemed that a comparative study of *Peranema* and *Heteronema* (both of the family Peranemidæ Klebs) and of several representative Astasiidæ might lead to results bearing on this problem. Some of the various difficulties, however, appear to have been settled satisfactorily in the classification proposed by Calkins (1926), although Rhodes (1926) has objected to the changes. In connection with work on several Astasiidæ and on *Heteronema,* this investigation was begun by Powell in 1925, and has been completed by the senior author at New York University. A brief account of our findings has been published elsewhere (Hall, 1926).

The nomenclature of *Peranema trichophorum* has undergone several changes since the organism was first described by Ehrenberg in 1838 as *Trachelius trichophorus*, a supposed ciliate. A few years later the organism was recognized as a flagellate by Dujardin (1841), and named *Peranema protracta*. Overlooking the work of Dujardin, Claparéde (cited by Kent) changed Ehrenberg's terminology to *Astasia trichophora*. The flagellate was replaced in the genus *Peranema* by Stein (1878), and is now known as *Peranema trichophorum* (Lemmermann, 1913; Conn and Edmondson, 1918; Calkins, 1926). It is to be noted that Conn and Edmondson (1918), although listing *Peranema trichophorum,* described an *Astasia trichophora* with two flagella, one long and the other relatively short. This organism, even if a valid species, cannot be regarded as an example of Kent's *Astasia trichophora* (= *Peranema trichophorum*), nor may it be called an *Astasia*, since a single flagellum is characteristic of this genus (Kent, Lemmermann, Calkins). The organism in question might be placed in either the genus *Heteronema* or *Distigma*, but reassignment would be rather difficult on the basis of Conn and Edmondson's figure.

MATERIAL AND METHODS.

For the past three years *Peranema trichophorum* has occurred persistently in various laboratory cultures at The Rice Institute and later at New York University. A satisfactory culture medium consists of tap or pond water containing a piece of beef suet. The medium is allowed to stand for a week or more, and then inoculated with *Peranema*.

Material has been fixed in the solutions of Altmann and Schaudinn. The latter is far more satisfactory for examination of the nucleus, pharyngeal-rod apparatus and flagellar rhizoplasts, while the fixative of Altmann gives good results with the flagellum and gullet, as well as with mitochondria (Causey, 1925). After Schaudinn's fixative material has been stained in Bordeaux red followed by iron-alum hematoxylin, and in iron-alum hematoxylin either alone or followed by a counterstain (eosin, orange G, Congo red). After fixation by Altmann's method Regaud's hematoxylin has been used, either with or without a counterstain (eosin or Bordeaux red). In other Altmann preparations the method of bleaching described by Causey (1925) has been tried for demonstration of mitochondria.

In observations on the living organism a Zeiss cardioid darkfield condenser has been used to advantage as a supplement to the usual microscopic examination. The darkfield condenser has been especially useful in determining the presence of cuticular striations and small granules (mitochondria?) which show Brownian movement, and in observing behavior of the flagellum. The use of a yellow color filter in place of the usual "daylight" glass of the microscope lamp was found to facilitate the tracing of chromosomes and rhizoplasts in material stained in Bordeaux red and hematoxylin. Our optical equipment consisted of a Spencer binocular microscope equipped with Zeiss compensating oculars and 2 mm. (Zeiss) and 1.5 mm. (Spencer) apochromatic objectives.

General Morphology.

Peranema trichophorum is a colorless, plastic uniflagellate euglenoid, varying in length from 22 to 70 μ, according to Lemmermann (1913). In our stained preparations the length of extended organisms ranges from 35 to about 60 μ. The living flagellate shows several peculiarities: a gliding type of forward locomotion, apparently without spiral rotation; a long and thick flagellum, only the tip of which seems to be involved in forward locomotion; marked plasticity, or metaboly, especially in food-taking or in changing direction of locomotion; and a pharyngeal-rod apparatus lying in the anterior part of the body. With the darkfield condenser, the periplast seems to be heavily striated and, as in *Jenningsia* (Schaeffer, 1918), the striations begin at the cytostome and extend in spirals posteriorly (Fig. *A*, 1). Occasionally the striations may be detected in both Altmann and Schaudinn preparations. There are no traces of "movable club-shaped appendages" such as are found on the periplast of *Jenningsia*. The cytoplasm usually contains a number of paramylum bodies and one or more food masses in vacuoles.

In Altmann-Regaud preparations of *Peranema* a number of small cytoplasmic granules are often to be observed, apparently more numerous in the outer layers than in the deeper cytoplasm. In some of our material fixed in Schaudinn's fluid and stained in Bordeaux red followed by hematoxylin, similar granules have been found; in such preparations the granules, when present at all, seem to be distributed throughout the cytoplasm. Such granules (Pl. I., Fig. 1; Pl. II., Figs. 10, 13) are possibly to be regarded as mitochondria, since they are readily demonstrated by Causey's (1925) methods of mitochondrial fixation and staining. It has been impossible, however, to demonstrate their mitochondrial nature by the use of Janus green B on living specimens. With the darkfield condenser granules of similar size are to be seen in the living organism and, so far as our observations go, they exhibit continuous Brownian movement. Distinctly rod-shaped mitochondria have not been found, and in this respect our findings agree with those of Causey (1926), who described only granular mitochondria in *Euglena gracilis.*

GULLET AND PHARYNGEAL-ROD APPARATUS.

In the living flagellate it has not been possible to determine completely the structure of the gullet and pharyngeal-rod apparatus. The following description of these structures is, therefore, based principally upon stained preparations, and secondarily upon confirmatory observations on living flagellates. The gullet (Fig. *A,* 2–5) is a more or less flask-shaped cavity opening to the outside through the cytostome. The somewhat enlarged posterior portion of the gullet is often designated as the *reservoir* (Calkins, 1926; Baker, 1926; Rhodes, 1926). Near the base of the gullet lies a large contractile vacuole, which is sometimes apparent in stained preparations. In watching this structure in several living organisms, the time between systoles was found to range from 10 to 15 seconds. The earliest stages of diastole could not be followed. Soon after systole, a single new vacuole appears, increases in size, and then discharges its contents into the gullet. A connecting duct could not be traced in living organisms, but indications of such a canal were found in a few Altmann-Regaud preparations. The flagellum, on entering the cytostome, passes posteriorly to end in the usual blepharoplast in the wall of the gullet (Pl. I., Figs. 1–3; Fig. *A,* 2, 3). This insertion may also be traced in the living organisms under oil immersion.

The pharyngeal-rod apparatus (Fig. *A,* 4, 5) extends along the wall of the gullet and ends at the rim of the cytostome. In the majority of organisms examined there are two distinct *pharyngeal-*

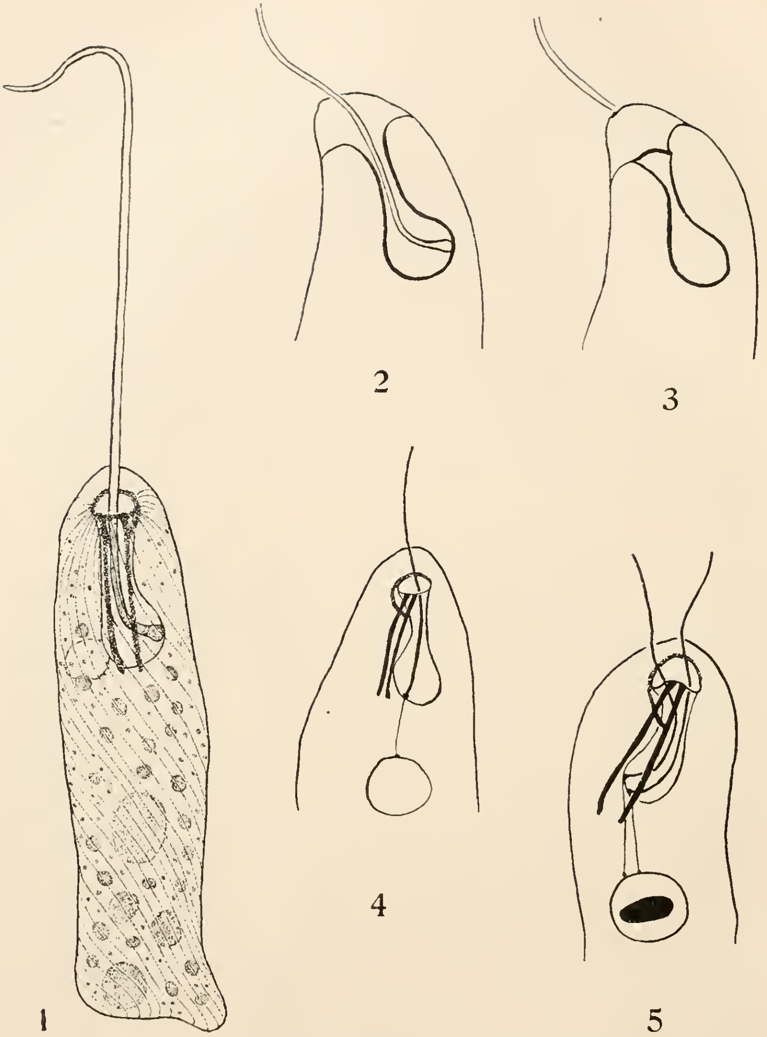

Fig. *A.* *Peranema trichophorum:* (1) diagrammatic sketch of extended organism showing flagellum, cytostome, gullet, contractile vacuole, pharyngeal-rod apparatus, spiral striations of periplast, nucleus, several food masses (posterior), paramylum bodies, and small granules which exhibit Brownian movement; drawing based upon living specimens and stained preparations; × 1500 approximately. (2) Gullet and cytostome in optical "frontal" section; pharyngeal-rod apparatus omitted; Altmann-Regaud preparation; × 2025. (3) The same organism, camera-lucida drawing of different focal plane to show "ventral" lip of cytostome; position of flagellum indicated beyond tip of body; × 2025. (4) Gullet, pharyngeal-rod apparatus and kinetic elements (blepharoplast, rhizoplast and centrosome); Bordeaux red-hematoxylin preparation; × 1570. (5) Pharyngeal-rod apparatus and kinetic elements in early prophase; two flagella, and two blepharoplasts joined by rhizoplasts to the daughter centrosomes; Bordeaux-red-hematoxylin preparation; × 1570.

rods, perhaps similar to the œsophageal rods of *Heteronema acus* (Rhodes, 1926). In other instances (Pl. I., Fig. 2) the rods are so close together that their double nature may be detected only at the ends; and in a number of our stained preparations it is possible to distinguish only a single thick rod (Pl. I., Fig. 4). Whether the occasional appearance of only one rod is due to fusion as a result of fixation is uncertain, but this does not seem improbable. Earlier descriptions of the pharyngeal-rod apparatus of *Peranema* have been discussed elsewhere (Hall and Powell, 1927). In addition to the pharyngeal-rods which lie alongside the gullet, another structure is sometimes to be detected in favorable preparations. This is a curved cytostomal element which, when it can be demonstrated, lies along one lip of the cytostome (Fig. *A*, 4, 5; Pl. I., Figs. 1, 3). From the lip of the cytostome the cytostomal rod extends in a slightly posterior direction around one side of the gullet to meet the pharyngeal rods. On the basis of Rhodes' (1926) description, the "falcate trichite" of *Heteronema acus* seems to be similar to this cytostomal element in *Peranema*. Furthermore, a curved rod of similar relationships is shown in Schaeffer's (1918) figures of *Jenningsia diatomophaga*.

The cytostome and gullet of *Peranema* function in the ingestion of solid food, which is formed into food vacuoles at the base of the gullet. The process of feeding has been described by Tannreuther (1923), who states that the protoplasmic contents of captured organisms are sucked in by way of the gullet, after the pharyngeal-rod has punctured the wall of the prey. Although confirmation of this description has been attempted repeatedly, such action of the pharyngeal-rod has so far escaped us. It has been found, however, that carmine particles, portions of plant cells and even entire organisms (apparently *Menoidium incurvum* and *Chilomonas paramecium*) are ingested, and that even in stained preparations there are evidences of the formation of food vacuoles at the base of the gullet. In watching living specimens in darkfield, it has been possible to observe the pinching off of food vacuoles from the base of the gullet. After their formation, the food vacuoles pass to the posterior half of the body. In its feeding habits, *Peranema* resembles Schaeffer's (1918) *Jenningsia* which, on account of its much greater size, is able to ingest diatoms as much as 100 μ in length. Contrary to the description of Tannreuther for *Peranema,* Schaeffer believes that in *Jenningsia* the pharyngeal-rods are not protruded in feeding.

The Nucleus.

In *Peranema trichophorum* the nucleus of the interphase contains an endosome which is often irregular in outline or fragmented (Fig. *B*), but commonly more or less ovoid in shape (Pl. I., Figs. 1, 2). In stained preparations there is usually a clear area immediately surrounding the endosome. Between the endosome and the nuclear membrane lie numerous chromatin granules, or chromomeres, apparently embedded in some sort of an achromatic network (Schaudinn and hematoxylin preparations). Bělař (1916) states that the endosome of *Peranema* is always alveolar in structure, even in the interphase. Such an appearance seems to be dependent upon methods of fixation and staining, since it is evident in some of our preparations but not in others. Hartmann and Chagas (1910) have described a divided "centriole" in the endosome of *Peranema* during the prophase, but they did not trace the structure through mitosis and the cytological evidence presented

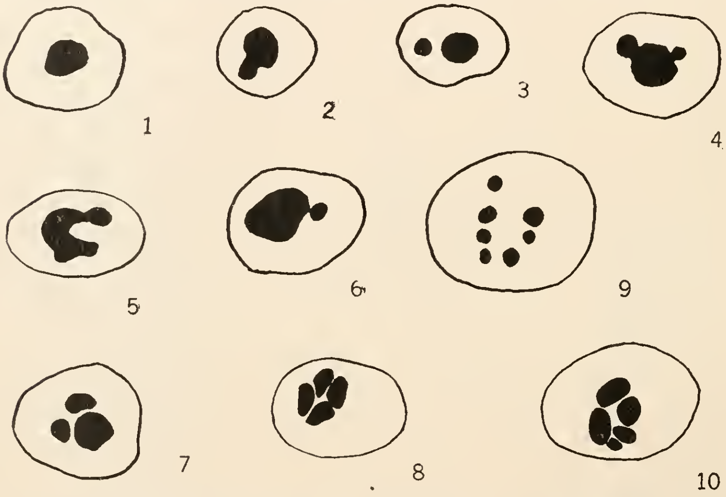

Fig. *B*. Camera-lucida drawings of nuclei to show variations in form of the endosome: 1–8, *Peranema trichophorum;* 9, 10, *Heteronema acus;* × 1045.

does not seem to justify their conclusions. In preparations fixed in Schaudinn's fluid and stained in iron hematoxylin without a counterstain, the endosome frequently seems to consist of a deeply staining central portion and a lighter peripheral layer. The darker medullary portion might conceivably be interpreted as a centriole. An interpretation of this sort, however, is confronted with the difficulty that the endosome (Fig. *B*) of *Peranema* is often fragmented. The two, three or four fragments may show the same peculiarity in staining with iron hematoxylin. Three or four such "centrioles" would seem to be too many for any one nucleus.

Nuclear Division.

The nucleus of the interphase is characterized by chromatin in the form of small granules, or chromomeres, surrounding the endosome and apparently embedded in the nodes of a lightly stained network. No definite chromosomes can be detected at this stage (Pl. I., Figs. 1, 2). In early prophases the chromatin granules are replaced by thread-like chromosomes (Pl. I., Fig. 3), formed presumably by fusion of these chromomeres. In some early prophases the chromosomes in their arrangement may simulate a spireme which, except for the greater number of chromosomes, is similar to that described in *Euglena agilis* (Baker, 1926). In others, the chromosomes appear to be radially oriented (Pl. I., Fig. 3). In later prophases, in which a second flagellum appears and division of the gullet takes place, indications of chromosome splitting are to be seen (Pl. I., Figs. 4–6). As in the case of *Euglena agilis* (Baker, 1926) separation of the daughter chromosomes begins, in the majority of cases, at one end of the pair and progresses toward the other end. This process results in the appearance of V-shaped structures, with a daughter chromosome forming each limb of the V (Pl. I., Figs. 5, 6). The V's gradually straighten out to form a belt of approximately parallel chromosome pairs (Pl. II., Figs. 8, 9), the members of each pair still being joined at one end (the "apex" of the V). This same type of chromosome splitting has been reported for *Menoidium incurvum* (Hall, 1923), as well as for *Euglena agilis*.

The endosome, during the later prophases (Pl. I., Figs. 5, 6; Pl. II., Figs. 8, 9), is gradually drawn out to form a central dumbbell shaped structure surrounded by the belt of chromosomes. Although the endosome of the interphase and earlier prophases frequently consists of two or more fragments, the elongating endosome of the later prophase is nearly always a single structure. In fact, only a single exception was found in our material; in this stage, a metaphase, there were two elongated endosomes, one slightly larger than the other. It seems possible, therefore, that separate endosomal fragments present in earlier stages may fuse to form a single mass before the late prophase.

In the metaphase (Pl. II., Fig. 10) the daughter chromosomes are completely separated in the equatorial plane of the elongated nucleus. During the anaphases (Pl. II., Fig. 11) the daughter chromosome belts draw apart as further elongation and constriction of the nucleus take place. The chromosomes still retain the parallel arrangement observed in the metaphase, and appear to be uniformly distributed around each half of the endosome. After constriction of the nucleus into two daughter nuclei in the early telophase the chromosomes may retain for some time their parallel arrangement (Pl. II., Fig. 12). Later on the chromosomes become irregularly distributed (Pl. II., Fig. 12), and finally chromomeres appear as reorganization of the daughter nuclei nears completion (Pl. II., Fig. 13).

The endosome, during metaphase and anaphase stages, becomes more and more elongated, so that the median portion is drawn out to form what has been called a *centrodesmose* (Calkins, 1926; Baker, 1926). The name centrodesmose is usually applied to a fibril joining the daughter halves of an intranuclear centriole. Since there is no evidence for the presence of a centriole in *Peranema,* the use of such a term will be avoided in this case. The median portion of the endosome finally breaks in the late anaphase just before the daughter nuclei are completely separated. For a time the daughter endosomes remain drawn out at their median ends (Pl. II., Figs. 11, 12). In later telophases, however, each endosome approaches the characteristic interphase form as reorganization of the daughter nuclei nears completion.

Kinetic Elements.

In the typical interphase (Pl. I., Figs. 1–2; Fig. *A*, 2) there is a single flagellum ending in a blepharoplast in the wall of the gullet. In the early prophase two blepharoplasts are apparent (Pl. I., Fig. 4), one of them giving rise to a short flagellar outgrowth, and the other serving for insertion of the old flagellum. At this stage the two blepharoplasts are still very close together. In slightly later prophases (Fig. *A*, 5) the flagellar outgrowth has increased in length, but the two blepharoplasts are still contiguous. The three stages just described are construed as indicating an early division of the old blepharoplast, followed by outgrowth of a new flagellum from one of the daughter blepharoplasts. This interpretation seems better supported by our evidence than is any hypothesis of nuclear origin of the blepharoplast (Baker, 1926). If the theory of nuclear origin of blepharoplasts were applied to *Peranema* it would necessitate the assumption that, in the earliest biflagellate stages observed (Pl. I., Fig. 4), a granule has already passed through the nuclear membrane and made its way forward, to become embedded in the wall of the gullet and give rise to a new flagellum. Such an assumption is not warranted on the basis of our observations. In the later prophases (Pl. I., Figs. 5, 6, 9) the two blepharoplasts with their attached flagella gradually draw apart as widening and fission of the gullet begin.

In interkinetic stages much difficulty has been experienced in attempting to trace a flagellar rhizoplast to the nuclear membrane, due to the fact that the interphase nucleus lies typically in the posterior third of the body. The food vacuoles and fixation vacuoles often intervening between nucleus and gullet (Pl. I., Fig. 1) make the search for a delicate rhizoplast extremely difficult. Although apparent rhizoplasts have been traced part way in a number of instances, it has been impossible to demonstrate to our own satisfaction a rhizoplast joining blepharoplast and nucleus during interphase stages. Such a rhizoplast, however, has been demonstrated in vegetative stages of another euglenoid, *Phacus costata* (Bretschneider, 1926), and it is not impossible that an existing interphase rhizoplast of *Peranema* has merely been overlooked by us. In the early prophase, however, it has been possible in a number of instances to trace a flagellar rhizoplast to the nucleus (Pl. I., Fig. 3; Fig. *A*, 4). In such cases the cytoplasm happened to be practically free from food vacuoles, so that the usual difficulties were not encountered. In later prophases (Fig.

A, 5) two rhizoplasts extend from the daughter blepharoplasts to a pair of granules on the nuclear membrane. This stage and the preceding one indicate that the extranuclear centrosome on the nuclear membrane divides during the prophase, and that each daughter centrosome is joined by a rhizoplast to the corresponding daughter blepharoplast. This arrangement of blepharoplast, rhizoplast and centrosome is found also in subsequent stages of binary fission (Pl. I., Fig. 5; Pl. II., Figs. 9, 11, 12). In flagellates in which the daughter gullets lie at different focal planes than the nuclei it is often difficult to trace rhizoplasts to the nuclei, and for this reason they are not indicated in some of our figures. Baker (1926) has described a similar rhizoplast joining each daughter blepharoplast to a so-called "parabasal homologue" at the nuclear membrane in *Euglena agilis*.

On the basis of our evidence it is impossible to prove that the granule, or centrosome, at the nuclear membrane is a permanent extranuclear organelle of *Peranema trichophorum*, on account of the difficulty in demonstrating a rhizoplast joining blepharoplast and centrosome in interkinetic stages. On the other hand, there is no evidence at all that the extranuclear centrosome ever arises from the endosome and migrates out of the nucleus. It is necessary, therefore, to leave the question open as to whether this extranuclear granule is a permanent structure (although so far undetected in interphase), or whether it may arise *de novo* at the beginning of binary fission.

Gullet and Pharyngeal-rod Apparatus.

In the early stages of binary fission the lower portion of the gullet gradually increases in width (Pl. I., Fig. 5). In such stages a pharyngeal-rod element is to be detected on each side of the gullet. This might suggest that one of the two original rods passes to each daughter organism. In the stage mentioned, however, the rods appear smaller than those of the typical vegetative stage. Furthermore, Rhodes (1926) states that during "division the old 'staborgan' (pharyngeal-rod apparatus) migrates posteriad and is resorbed" in *Heteronema acus*. "In the early telophase new ones differentiate from the cytoplasm." Rhodes' interpretation might be supported by our Fig. 5 of *Peranema*, in which rod-like structures are shown enclosed in two food vacuoles posterior to the nucleus. Even in this stage, however, pharyngeal-rod elements are apparent on either side of the gullet. Hence, if replacement of the apparatus does occur in *Peranema,* as might be expected, the process must take place rapidly and at some time before late prophase. Division of the gullet is completed in late prophase (Pl. I., Fig. 7). In metaphase and following stages the lower ends of the two gullets lie near the poles of the elongated nucleus and later at the poles of the daughter nuclei, polarity being indicated by the orientation of the endosome or its daughter halves. Separation of the two gullets seems to begin always at the lower end and to progress anteriorly.

Origin of the Centrosome in Mastigophora.

An extranuclear division center is apparently of common occurrence in the Mastigophora, since such a structure has been described or figured in one or more members of each of the following families (Calkins' system): Ochromonadidæ (Chrysomonodida); Protodiniferidæ, Noctilucidæ, Blastodiniidæ, Peridiniidæ (Dinoflagellida); Euglenidæ, Astasiidæ (Euglenida); Rhizomastigidæ (Pantastomatida); Choanoflagellidæ, Bodonidæ (Protomastigida); Tribes Monozoa and Diplozoa (Polymastigida); Lophomonadidæ, Staurojoenidæ, Trichonymphidæ and Holomastigotidæ (Hypermastigida). On the basis of structural relationships, avoiding consideration of the highly specialized Hypermastigida, two general types of extranuclear division centers may be recognized in the flagellates.

The *centroblepharoplast* (Kofoid), or centrosome-blepharoplast (Minchin), combines in a single structure the functions of both centrosome and blepharoplast. In the interkinetic phases (Fig. *C,* 1) the flagellum arises from the centroblepharoplast. In nuclear division the centroblepharoplast divides, and the daughter granules take up positions at opposite poles of the nucleus (Fig. *C,* 2), thus resembling in behavior the centrosomes in Metazoan mitosis. There is of course need for caution in interpreting such structures in flagellates, since the granules are often small and are not always easy to demonstrate. Since morphology and staining reactions alone are clearly inadequate for confirmatory evidence, it seems that the behavior of such a granule during nuclear division constitutes the only practicable basis for determining its centrosomal nature in flagellates. In accordance with this assumption, a blepharoplast which divides early in binary fission, and whose daughter blepharoplasts appear and remain at opposite poles of the nucleus throughout mitosis, may be regarded as a centrosome. To such a blepharoplast, therefore, the term *centroblepharoplast* is to be applied in the sense just explained. As a matter of fact, there would seem to be no logical objection to the view that a centrosome may exhibit the properties of a blepharoplast, in view of the behavior of the centrosome in spermatogenesis in most Metazoa. The following are some of the flagellates in

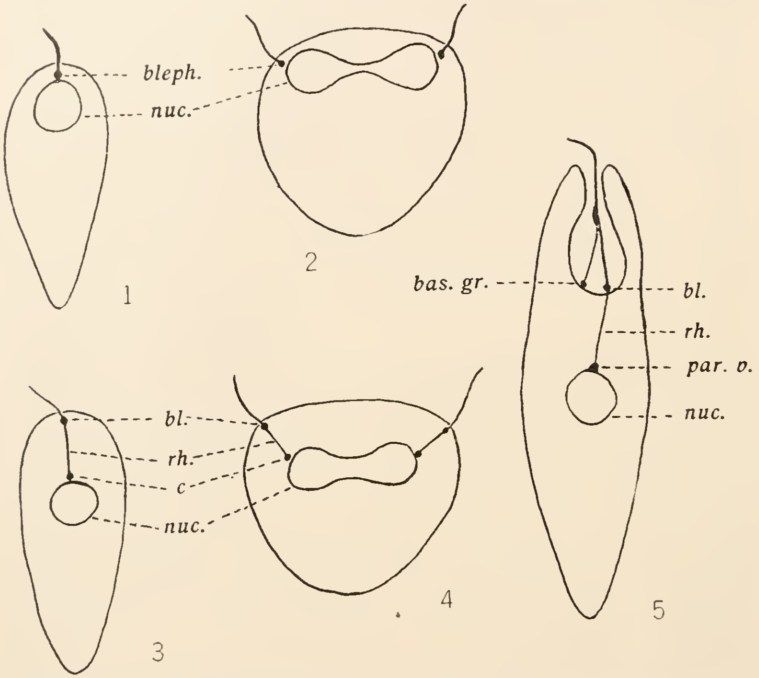

Fig. *C*. (1) Centroblepharoplast of interkinetic stages in flagellate; (2) centroblepharoplast in binary fission; (3) interkinetic stage, separate blepharoplast and extranuclear centrosome; (4) binary fission, separate blepharoplast and centrosome; (5) Baker's (1926) concept of kinetic elements in *Euglena agilis* (after Baker). *bas. gr.*, basal granule ("basal body"); *bl.*, blepharoplast; *bleph.*, centroblepharoplast; *c.*, centrosome; *nuc.*, nucleus; *par. b.*, parabasal homologue (Baker); *rh.*, rhizoplast.

which a division center of this type has been described: *Eutrichomastix serpentis, Trichomonas augusta, T. muris* (Kofoid and Swezy, 1915), *T. muris* (Wenrich, 1921), *T. batrachorum, Monocercomonas melolonthæ* (Grassé, 1926), *Heteromita uncinata* and *Cercomonas longicauda* (Wenyon, 1926).

In the second type, *centrosome* and *blepharoplast* appear to be two separate structures. In a number of flagellates, for example, a rhizoplast extends from the blepharoplast (or blepharoplasts) to a granule, designated as the extranuclear centrosome, on the nuclear membrane (Fig. *C*, 3). At an early stage of binary fission this centrosome divides, as may also the blepharoplast and rhizoplast, and the daughter centrosomes move to opposite poles of the nucleus (Fig. *C*, 4). In some instances, each daughter centrosome is joined by a rhizoplast to a corresponding blepharoplast. In other forms, such as *Giardia*, only one of the centrosomes may be connected with a blepharoplast. A few of the flagellates in which a division center of the second type has been described are: *Giardia* (Kofoid and Christiansen, 1915; Kofoid and Swezy, 1922), *Menoidium incurvum* (Hall, 1923), *Polytomella citri* (Kater, 1925), *Proteromonas longifila* (Grassé, 1926), and possibly *Euglena agilis* (Baker, 1926). In addition to the centrosome-rhizoplast-blepharoplast arrangement, some workers have described or figured granules which appear at the poles of the dividing nucleus but do not, apparently, show rhizoplast connections with the blepharoplasts. While such granules are more liable to misinterpretation than those which are undoubtedly joined to the blepharoplasts, it seems probable that at least some of them may be of centrosomal nature.

In the case of the centroblepharoplast of many flagellates it is possible, with certain obvious reservations, to regard the centrosome as a permanent organelle which functions as a component of the locomotor apparatus in vegetative stages and, in addition, as a division center in binary fission. This interpretation, with the same reservations, might be applied to flagellates in which centrosome and blepharoplast are separate structures, provided a rhizoplast can be traced from blepharoplast to centrosome during interkinetic as well as division stages. Such a concept of continuity of the extranuclear centrosome is, however, confronted with the objection that little is known of the fate of this structure during encystment in flagellates. In *Chilomastix mesnili* (Kofoid and Swezy, 1920), a form characterized by nuclear division within the cyst, a division center of the second type appears in encysted stages. This is true also of *Giardia enterica* (Kofoid and Swezy, 1922). As for flagellates which do not divide within the cyst, there is little critical evidence as to whether the centrosome persists or not. Furthermore, any attempt to solve this problem is handicapped by the fact the the criterion of behavior during nuclear division cannot be employed for identification of a suspected centrosome. In *Polytomella citri*, however, Kater (1925) has demonstrated the presence, just prior to excystment, of an extranuclear granule joined by rhizoplasts to the blepharoplasts (from which flagella subsequently grow out). Continuity of the centrosome during encystment might be suggested by the cases cited, but it is impossible without more evidence, to draw any definite conclusion.

When a connection between blepharoplast and extranuclear centrosome cannot be traced in interkinetic stages, there arises the problem as to how and where the centrosome originates at the onset of binary fission. Several possibilities are apparent. (1) The centrosome may arise from within the nucleus, becoming extranuclear at the beginning of nuclear division. (2) The centrosome may be permanently extranuclear, but easily detected only in stages of mitosis. (3) The extranuclear centrosome may arise *de novo* in the cytoplasm at the beginning of nuclear division, and then disappear after the completion of binary fission.

From various descriptions of nuclear origin of extranuclear kinetic granules, it would appear that the typical process is as follows. A small outgrowth first appears on one side of the endosome. This endosomal bud breaks off and moves toward the nuclear membrane, finally passing through it to become an extranuclear granule. Once in this position, the granule may bud off blepharoplasts or basal bodies, give rise to flagella, or otherwise assert its kinetic nature. Three similar descriptions of such a process may be mentioned. One of the earlier accounts is that of Wilson (1916), who found that in *Dimastigamœba* (*Nægleria*) *gruberi* the blepharoplast seemed to grow out from the endosome of the nucleus at the beginning of enflagellation. Wenyon (1926), after a critical study of this species, states: "The writer, after examining many thousands of amœbæ at all stages of flagellum formation, has failed entirely to trace the origin of the blepharoplasts from the karyosome. . . . It seems more probable that the blepharoplasts are present in the cytoplasm even during the amœboid phases of the organism. They are so minute that, unless their connection with the flagella can be traced, it is impossible to distinguish them from other granules which occur in the cytoplasm. . . . There seems to be no real evidence that they are derived from the karyosome of the nucleus."

In a more recent paper Kater (1925) has described such an origin of the centrosome in *Polytomella citri* just before excystment takes place. At a late stage of encystment a small bud appears on one side of the endosome. In the next step, two chromatic masses are shown in the nucleus, one the central karyosome and the other a "chromatic ball" just within the nuclear membrane. In the third stage, a granule ("chromatic ball") is shown immediately outside the nuclear membrane. Later, a small "basal granule" separates from the chromatic ball and moves toward the periphery of the cell, remaining connected by a rhizoplast with the parent granule. In the later stages, the basal granule and rhizoplast divide and, at the beginning of excystment, flagella grow out from the basal granules.

Kater's paper is open to the following criticisms. His critical stage (Fig. 35), in which an intranuclear granule is shown at the periphery of the nucleus, offers no proof that such a "chromatic ball" is on its way to passage through the nuclear membrane. It is possible that this "chromatic ball" may be nothing more than a fragment of the karyosome, since Kater's figures show that the karyosome breaks up in early division stages. In addition, the mere presence of an extranuclear granule on the nuclear membrane (Kater's Fig. 36) is in itself no evidence at all that such a position is the result of a migration out of the nucleus. Furthermore, the fact that a fibril *apparently* joins the karyosome to this extranuclear granule means, in this instance, precisely nothing, since the typical interphase nucleus of *Polytomella* shows from four to six such fibrils extending from the karyosome to the nuclear membrane. Most important of all, Kater has not proved that the "chromatic ball" (centrosome) is not permanently extranuclear during encystment. He actually concludes that, in the active flagellates, the extranuclear centrosome is present in interkinetic as well as in division stages. In the encysted forms Kater figures in several stages a large number of "metachromatic granules" closely surrounding the nucleus. Even if the "chromatic ball" were extranuclear in such cases, it could not be detected. In presumably later stages of encystment the metachromatic granules have migrated to the periphery of the cell, and the "chromatic ball" is revealed just outside the nuclear membrane.

Baker (1926) has described a similar phenomenon in *Euglena agilis:* "One of the first evidences of approaching mitosis is the budding off from the endosome of a deeply staining granule of unmistakable character. . . . The bud increases in size . . . and passes . . . through the chromatin of the outer nucleus to the nuclear membrane. . . . On the nuclear membrane the mass divides and the daughter bodies separate and pass gradually to opposite sides of the nucleus. . . . As they separate the bodies again divide giving rise to the blepharoplasts of the two future cells. From each blepharoplast a new flagellum arises. When the nucleus has passed anteriorly until it comes in contact with the lower border of the reservoir, each blepharoplast divides, giving rise to a basal granule which serves as the basis for the smaller branch of the bifurcated flagellum. . . . The mass budded from the endosome contains blepharoplasts, basal bodies, and a chromatic residue . . . (which) remains on the nuclear membrane."

In an early prophase (Baker's Fig. 3) a small darkly stained granule is shown near the endosome and attached to it by a rhizoplast. In succeeding stages (Figs. 4, 5) two granules (supposedly division products of the first), one of them still joined to the endosome, are to be seen near the nuclear membrane. These two granules look surprisingly like several other granules in the nuclei of Figs. 4 and 5. In Fig. 6 there are two pairs of extranuclear granules at opposite sides of the gullet. Each pair of granules is said to represent a blepharoplast and basal body to which "each daughter half of the kinetic complex has given rise." The "daughter halves of the kinetic complex" are no longer to be seen on the nuclear membrane. Instead, a single granule ("residue of the kinetic complex ") invested with a halo is adjacent to and joined by a rhizoplast to the endosome.

And yet it is stated that each half of the kinetic complex gives rise to a " blepharoplast-basal body complex which becomes the base of the bifurcated flagellum " and to a chromatoid residue which "*remains on the nuclear membrane.*" After reading this statement, careful examination of Baker's figures 3 to 8 leaves the reader somewhat puzzled. There is no *apparent* difference between the " kinetic complex " of the early prophase (Fig. 3) and the " residue of the kinetic complex " shown near the endosome in Fig. 6; in fact, the figures would even suggest identity of the two structures. How then is it possible to assume that between these two similar stages the kinetic complex has given rise by division to *two daughter granules on the nuclear membrane,* and that each of these granules has budded off an extranuclear blepharoplast-basal body, leaving a chromatoid residue *on the nuclear membrane.*

In Fig. 8 the daughter blepharoplasts are seen at opposite ends of the nucleus. One of the blepharoplasts is now connected with the single residue of the kinetic complex, which lies near the endosome. In Figs. 9, 10 and 11 the single residue of the kinetic complex has apparently disappeared, and the blepharoplasts are no longer shown on the nuclear membrane. Instead, two granules lie at the " poles " of the nucleus, while the blepharoplasts occupy their normal positions in the wall of the gullet. Each of these new granules, joined by a rhizoplast to its respective blepharoplast, is designated as a " residue from the kinetic complex." Have the two residues of Fig. 9 arisen by division of the single residue near the endosome in Fig. 8? Baker fails to make this clear. During the telophases each " residue " pinches off from the nucleus in a little nimbus of its own and loses connection with the blepharoplast. Each residue persists until some time during the next mitosis, and while " it does not function as a division center, yet it sets up a series of concatenated events which result in the final production of two fully vigorous individuals from a single parent cell."

Baker's description does not explain: (*a*) the reason for the striking similarity between the kinetic complex in Fig. 3 and the single residue of the kinetic complex in Fig. 6 and 8; (*b*) the relation between the single residue near the endosome in Figs. 6 and 8 and the two residues on the nuclear membrane in Fig. 9 and following stages; (*c*) the shifting of the blepharoplasts from the poles of the nucleus in Fig. 8 to their usual position (Fig. 9, etc.); (*d*) the fact that in Fig. 8 a rhizoplast is traced from one blepharoplast (on the nuclear membrane) to the single residue near the endosome, whereas in Fig. 9 each blepharoplast (now in the wall of the gullet) is joined by a rhizoplast to a residue at one pole of the nucleus; or (*e*) the fact that, while the chromatoid residues are said to remain on the nuclear membrane, a single residue is shown near the endosome in Figs. 6 and 8. The only possible conclusion seems to be that the evidence presented fails to prove that the extranuclear blepharoplast and basal body are derived from the intranuclear kinetic complex. No critical stages of such a process are shown in the plates and, on the basis of the evidence presented in Figs. 1 to 9, Baker's hypothesis seems to be no more than the following assumption: given two intranuclear granules in an early prophase and four extranuclear granules in a later prophase, the extranuclear granules must have been derived from the intranuclear ones. Furthermore, the plates offer no cytological proof that the polar chromatic residues (also termed "centroblepharoplast homologue" and "parabasal homologue") are actually derivatives of the kinetic complex. Since Baker's hypothesis of nuclear origin of kinetic granules is based entirely upon the supposed demonstration of the two phenomena just mentioned, a demonstration which his plates fail to show, his account cannot be accepted until verified.

In the case of *Peranema trichophorum* it is perhaps significant that the examination of several thousand interkinetic and early division stages has failed entirely to reveal any positive evidence for nuclear origin of extranuclear kinetic granules.

In conclusion it may be pointed out that the various workers cited have presented no critical evidence that the centrosome or blepharoplast is derived from the intranuclear endosome in flagellates. Wilson's description has been refuted by Wenyon, the evidence in Kater's paper is entirely inadequate, and Baker's plates do not contain the proof for his hypothesis. Various other accounts of nuclear origin of kinetic granules, mentioned by Calkins (1926) and Wenyon (1926), seem to be no more convincing. There is no need to point out that most of the evidence against the theory of nuclear origin is purely negative in character, but in spite of this fact the whole doctrine of nuclear origin of kinetic granules in flagellates can only be regarded as "not proven." And until adequate proof is forthcoming there seems to be little justification for the statements of various authors that there exists, hidden away in the endosome of interkinetic flagellates, an intranuclear centriole, or endobasal body, which gives rise to blepharoplasts, centrosomes or other kinetic elements during binary fission.

The Parabasal Homologue Concept.

In the paper just discussed Baker (1926) has introduced another complication in the concept of a homology between his "residue of the kinetic complex" and the parabasal body. "An endobasal body is located in the endosome and gives rise by division to a chromatoid mass, the kinetic complex. This migrates to the periphery of the nucleus, where it divides, the daughter halves passing to the approximate poles of the division figure. . . . The chromatoid residue left on the nuclear membrane after the blepharoplast-basal body complex is freed . . . is homologous to the parabasal body of many parasitic flagellates as well as to the so-called kinetonucleus of Trypanosomes." There is no evidence in Baker's figures for the existence of an *endobasal body* within the endosome, or for the formation of the kinetic complex by division of such a pre-existing granule in the endosome. Obviously, the endobasal body must be a purely hypothetical structure in this case.

The parabasal homology of the "residue of the kinetic complex" is said to be indicated partly by the position of the residue with relation to the poles of the dividing nucleus. It is impossible to accept such a statement without reservation. In Bělař's (1926) figures of nuclear division in *Trypanosoma loxiæ,* for example, the position of the daughter parabasal bodies is extremely variable with respect to the poles of the nucleus. In other parasitic flagellates, such as *Giardia* and *Trichomonas,* the centrosomes (or centro-blepharoplasts), and not the parabasal bodies, occupy the poles of the nucleus.

Another foundation for Baker's concept is his apparent belief that the kinetic residue and parabasal body show the same sort of connections with the nucleus and blepharoplast. Thus he stresses the view that in trypanosomes and crithidial forms the parabasal body is joined to the nucleus by a rhizoplast. As a matter of fact, McCulloch (1919) has demonstrated that in *Crithidia euryophthalmi* and *Schizotrypanum cruzi,* flagellates mentioned by Baker, this is not the case. Instead, the nuclear rhizoplast extends from the blepharoplast to the nucleus, while the parabasal body is joined by its own rhizoplasts to the blepharoplast and not to the nucleus. In other flagellates as well (*e.g., Trichomonas, Polymastix, Giardia*), the parabasal body seems to be connected with a blepharoplast rather than with the nucleus.

Another supposed support for this hypothesis is the statement that "McCulloch (1917) described the origin of a chromatoid mass from the endosome in *Crithidia euryophthalmi.* . . . She identifies the structure as the parabasal body." An examination of McCulloch's article shows that nowhere in the paper cited does she mention such an origin of the parabasal body. Nor does she, in her other papers (1915, 1919), figure or describe the origin of the parabasal body directly from the nucleus. It would seem that Baker, instead of reading carefully McCulloch's original papers, allowed himself to be misled by Calkins (1926). On page 97, Fig. 46, of his textbook, Calkins has copied several figures of small zooids of *Crithidia* and labeled them "origin of parabasal from endosome of nucleus (after McCulloch)." This interpretation is somewhat liberal, since McCulloch (1919) herself suggested a nuclear origin of the blepharoplast, from which in later development a flagellum "grows forward anteriorly and a parabasal body to the side." On the basis of McCulloch's statements, it would be just as logical to conclude that the flagellum originates from the endosome within the nucleus as to assume that the parabasal body is budded off directly from the endosome. It might be mentioned also that McCulloch qualified her suggestion with the statement that her search for a complete series of stages in the origin of blepharoplast and parabasal body had been disappointing; and that, "owing to the size of these zooids it is extremely difficult . . . to form any adequate conception of their structure." Furthermore, the belief in nuclear origin of the parabasal body is in opposition to Wenyon's (1926) opinion that "there is no conclusive evidence that the parabasal body is of nuclear origin, as some have supposed."

Baker's parabasal homologue concept thus rests in part upon an apparent misunderstanding of the relationships of the blepharoplast, parabasal body and nucleus in *Crithidia* and other flagellates, and in part upon the seemingly erroneous impression that the parabasal body of *Crithidia euryophthalmi* is budded off directly from the endosome. On the basis of Baker's figures of *Euglena agilis* it is evident that the so-called "parabasal homologue" is similar in behavior, for the most part, to the extranuclear centrosome in a number of the flagellates already mentioned. For this reason it would seem a more plausible interpretation to consider this "homologue" a centrosome joined by a rhizoplast to the blepharoplast, rather than a rudiment of the parabasal body of parasitic flagellates.

SUMMARY.

Peranema trichophorum is a colorless, metabolic euglenoid with a single large flagellum, only the tip of which is employed in forward locomotion. The flagellum arises from a blepharoplast in the wall of the flask-shaped gullet and leaves the body through the cytostome. The pharyngeal-rod apparatus ("staborgan") consists typically of two pharyngeal-rods and a curved cytostomal element. The rods extend from the rim of the cytostome posteriorly along the gullet. The cytostomal element lies along one "lip" of the cytostome and extends partly around the gullet to join the pharyngeal-rods. The apparatus probably operates in expansion of cytostome and gullet in feeding, although this could not be determined in living material with any degree of accuracy.

In mitosis longitudinal splitting of the chromosomes occurs, followed by unipolar separation of the daughter chromosomes. This results in the appearance of V-shaped daughter chromosome pairs, which unfold to form the chromosome belt of the later prophases. Final separation of the daughter chromosomes (at the "apex" of each V) occurs in the metaphase. This process is similar to that described in *Menoidium incurvum* (Hall, 1923) and *Euglena agilis* (Baker, 1926).

The kinetic elements consist of a blepharoplast, in which the flagellum ends, a centrosome on the nuclear membrane, and a rhizoplast joining blepharoplast and centrosome. The blepharoplast-rhizoplast-centrosome complex was detected in early prophases, but not in interkinetic stages. In later prophases both centrosome and blepharoplast apparently divide, and the daughter centrosomes gradually move apart toward opposite poles of the nucleus. A second flagellum grows out from one of the daughter blepharoplasts. There is no evidence for nuclear origin of centrosome or blepharoplast in *Peranema trichophorum.*

The question of the nuclear origin of centrosome and blepharoplast in flagellates is discussed, with special referenence to the papers of Kater and Baker. It is pointed out that neither paper contains sufficient evidence to prove a nuclear origin of kinetic elements.

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DESCRIPTION OF PLATES.

Peranema trichophorum: drawings from preparations fixed in Schaudinn's fluid and stained in Bordeaux red followed by iron-alum hematoxylin; magnification of 1570, except Fig. 8.

Plate I.

Fig. 1. Nucleus in interphase; cytoplasm contains several food masses in vacuoles near nucleus; cytoplasmic granules (mitochondria?) are numerous.

Fig. 2. Contracted organism; nucleus in interphase; pharyngeal rods appear separate only at anterior ends.

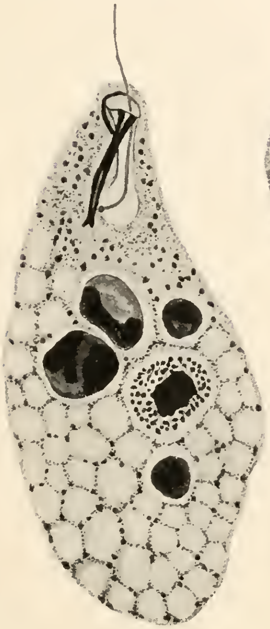
Fig. 3. Early prophase; rhizoplast extends from blepharoplast to centrosome at nuclear membrane; two endosomal fragments; chromosomes show radial arrangement.

Fig. 4. Prophase; two blepharoplasts present, and a new flagellar outgrowth has appeared; rhizoplasts not traced to nucleus (see figure *A*, 5), since the latter lies at a deeper focal plane than the gullet; only one thick pharyngeal-rod to be seen; some indications of chromosome splitting.

Fig. 5. Prophase; optical section of gullet and cytostome; position of pharyngeal-rods indicated; separation of daughter chromosomes beginning.

Fig. 6. Later prophase; V-shaped chromosome-pairs evident; portion of one daughter flagellum and gullet indicated.

Fig. 7. Different focal plane through same organism; the two daughter gullets have just separated anteriorly; two pairs of pharyngeal-rods present.



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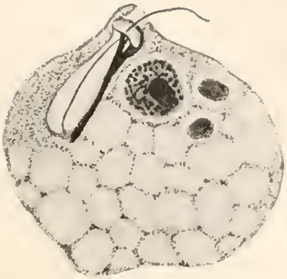

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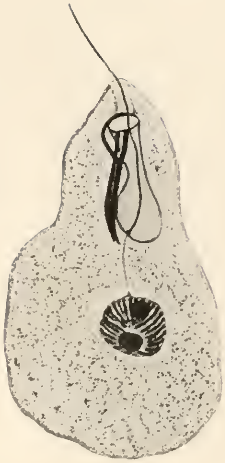

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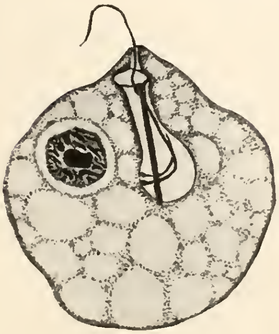

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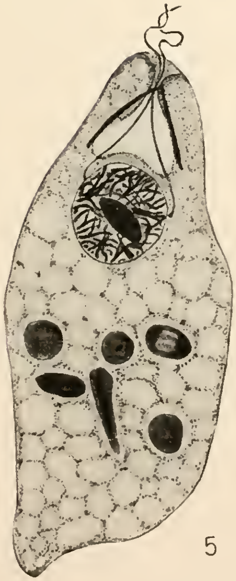

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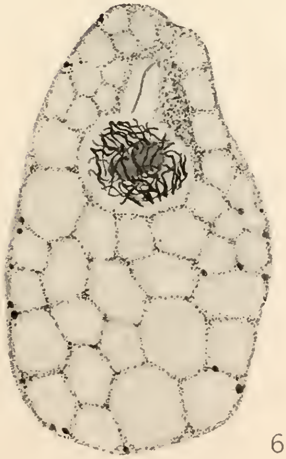

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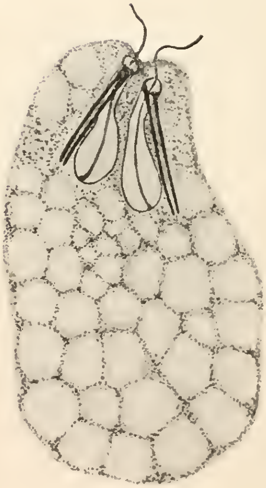

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PLATE. II.

FIG. 8. Late prophase; daughter chromosome pairs unfolding to form chromosome belt; the gullets, which lie almost perpendicular to the plane of the drawing, are omitted; × 2025.

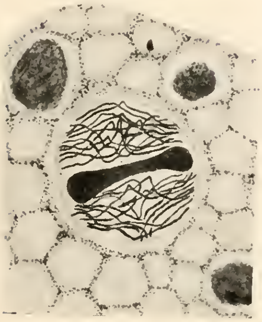
FIG. 9. Late prophase; chromosome-V's almost completely unfolded to form chromosome ring around the elongated endosome; right gullet in optical section; left gullet and flagellum, which extend beneath the nucleus, indicated in their approximate positions; two pairs of pharyngeal-rods apparent.

FIG. 10. Metaphase; daughter chromosomes completely separated in equatorial plane; the gullets, lying at deeper focal plane than nucleus, are shown in optical section; pharyngeal-rod apparatus omitted in order to show distribution of granules (mitochondria?).

FIG. 11. Late anaphase; daughter chromosome groups and daughter endosomes well separated; nucleus almost completely constricted; gullets shown in optical section; rhizoplast shown joining one blepharoplast to a centrosome.

FIG. 12. Telophase; in one daughter nucleus the chromosomes still show a parallel arrangement, in the other they are becoming irregularly distributed; from each blepharoplast a rhizoplast extends to a centrosome at the nuclear membrane; on the left only the lower end of the gullet is shown, and the pharyngeal rods are omitted.

FIG. 13. Later telophase; daughter nuclei undergoing reorganization; gullets, which extend beneath the nuclei, are indicated in optical section; two pairs of pharyngeal-rods apparent.



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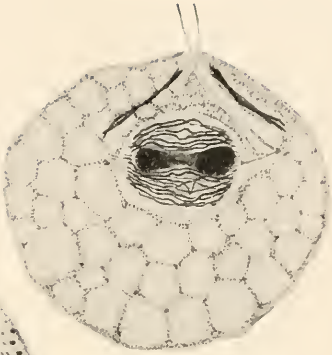

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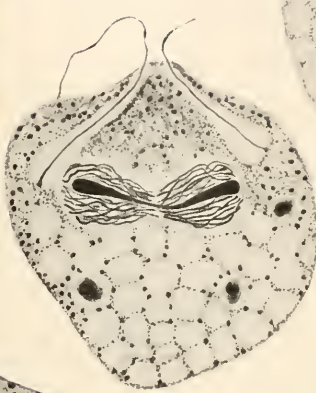

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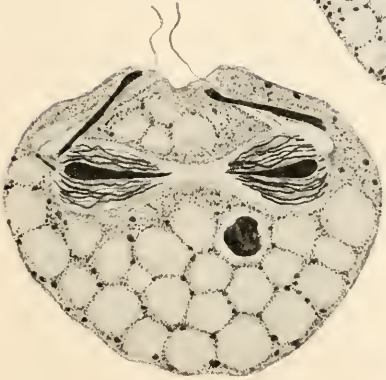

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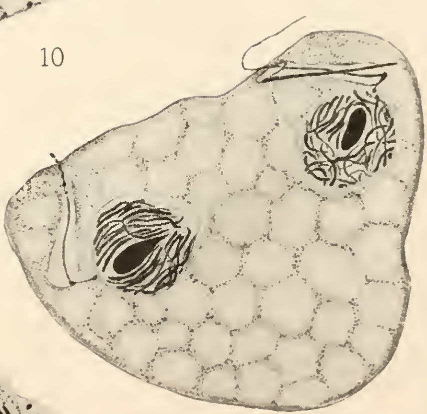

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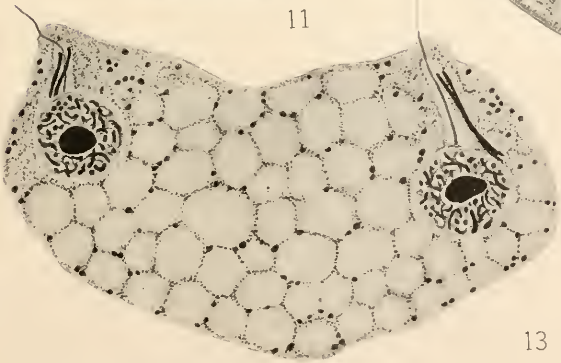

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MISCELLANEOUS OBSERVATIONS ON *HYDRA*, WITH SPECIAL REFERENCE TO REPRODUCTION.

LIBBIE H. HYMAN,

Hull Zoölolgical Laboratory, University of Chicago.

The following observations have accumulated during several years' cultivation of hydra in the laboratory together with numerous collections of the animals from nature.

The taxonomy employed is that of Schulze ('17) who has made a much needed revision of the hydras and furnished adequate descriptions of the known species. He divides them into three genera: *Chlorohydra*, with symbiotic algæ in the entoderm; *Pelmatohydra*, column differentiated into body and stalk; and *Hydra*, sensu strictu, column not so differentiated. The common green *Hydra* then becomes *Chlorohydra viridissima* (= *Hydra viridissima* Pallas, *Hydra viridis* Linnæus); the brown *Hydra* is designated as *Pelmatohydra oligactis* (= *Hydra oligactis* Pallas, *Hydra fusca* Linnæus, *Hydra diœcia* Downing); and the genus *Hydra* contains a number of species some of which in the past have been confused under the name of *Hydra vulgaris* (= *Hydra grisea* Linnæus). There are at least four, possibly five, species of hydra around Chicago, of which one does not correspond with any of Schulze's descriptions and requires to be named and described.

The observations in this paper concern *Pelmatohydra oligactis* (Pallas) Schulze and *Hydra stellata* Schulze.

Cultivation.

The cultivation of hydras in the laboratory is not difficult, provided sufficient food is available. The real problem is therefore the maintenance of an adequate food supply. *Daphnia* or other daphnids furnish the most convenient food and are raised as follows. Several dishpans or tubs are partly filled with well-aërated water. To each is added a sufficient amount of boiled aquatic vegetation such as *Elodea* or *Ceratophyllum* to cover the bottom with a thin layer of debris without inducing visible fermentation. The water should remain clear. After such cultures have stood for three or four days, dapnids are added to them. Later small pieces of fresh raw liver or the freshly killed bodies of small vertebrates are added but never in sufficient amount to cause cloudiness of the water. Each such culture serves for some time but new cultures must be prepared at intervals.

Hydras collected from nature are transferred to cultures prepared as follows.[1] Jars are filled one-half to three-fourths full of thoroughly aërated water (avoid water that has been treated with germicides). To each jar is added a small amount of water plant such as *Elodea* that has been boiled for ten minutes. My observations agree with those of Goetsch ('22*b*) that hydras flourish better in cultures containing some organic debris than in clear water. After jars so prepared have stood for several days, remaining clear and not showing any visible signs of fermentation, hydras may be added to them. Such cultures of hydra must be fed daily by adding an appropriate number of daphnids to each. One daphnid per hydra per day is sufficient to keep a culture in good condition but to induce rapid budding each animal must receive three or four daphnids daily. At frequent intervals the ejected remains of daphnids must be removed from the bottom of the culture and a small amount of water dipped out and replaced by prepared culture water.

It frequently happens that freshly collected hydras undergo depression (see below) after a short sojourn in the laboratory and are apt to die, owing probably to the sudden change of environmental conditions. It may therefore be often difficult to start laboratory cultures from specimens taken from nature. Under such circumstances it is necessary to resort to "hand-feeding" to keep the animals alive until they have become adjusted to laboratory conditions. By "hand-feeding" one means that each hydra is fed daily by placing a crushed daphnid or *Cyclops*, preferably the latter, in contact with its tentacles by means of a fine forceps. After several days of such care, some specimens will usually have

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[1] The expression "prepared culture water" or "culture water" used hereafter refers to water prepared in the manner here described.

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recovered and will serve for starting cultures. At intervals new cultures should be prepared and hydras transferred to these. In my experience, *Pelmatohydra oligactis* is much easier to cultivate than any other species.

The chief difficulty encountered in the continuous cultivation of hydra in the laboratory is the occurrence of the condition known to German zoölogists as depression.

DEPRESSION.

This phenomenon, well known to European investigators, has received almost no attention from American zoölogists.[1] It had been seen by many of the early students of hydra, beginning with Trembley, but was first adequately described by R. Hertwig ('06) who also suggested the name depression, derived from supposedly similar phenomena in Protozoa. Later Hertwig's student Frischholz ('09) published an extensive paper on the matter and since that time several workers have considered the phenomenon.

I have observed depression on numerous occasions in various species of hydra, particularly in *P. oligactis*. The first symptom of the onset of depression is a refusal to feed accompanied by a persistent slight contracted state of column and tentacles. The tentacles then gradually disappear from the tips proximally. Meantime, column and tentacles continue to shorten, presenting a stout, stiff, contracted appearance. Soon the animal is reduced to a short stout cylindrical body bearing short stumps of tentacles. At this stage or earlier, the animal frequently ceases to maintain an erect position, often lying on its side; the basal disk may lose its hold on the substratum. As depression continues, the tentacles completely vanish and the greatly shortened column diminishes from its distal end towards the basal disk. At length only a small round ball, constituting the basal end of the hydra, remains and this soon disintegrates. Hydra may recover spontaneously from early stages of depression or may be induced to recover by altering environmental conditions; but recovery from advanced stages of depression seems to be impossible.

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[1] An example of this is furnished by the recent paper of Kepner and Jester ('27). Finding specimens of the green hydra with shortened tentacles they ascribe this to an ingestion of its own tentacles by the animal. While this apparently does occasionally happen it is obvious from their description and figures that the majority of these specimens with shortened tentacles as well as those with incomplete tentacles which they found in nature are simply in a state of depression. Depression and starvation are independent phenomena and the shortening of the tentacles in depression has no relation to starvation. The ejection of cells and fragments from the mouth is characteristic of animals in early stages of depression. The finding of nematocysts in the entoderm cells is no proof that ingestion of tentacles had occurred since in depression the cells pass into the interior.

The first satisfactory account of the histological changes occurring during depression is that recently given by Rehm ('25). According to Rehm, depression begins in the entoderm cells which gradually become loose and pass into the gastrovascular cavity. The shortened swollen appearance of tentacles and column in depression is due to the accumulation within the cavity of loose entoderm cells which may also be discharged from the mouth in early stages. Later the mouth closes. After the entoderm has largely disintegrated, the mesoglœa dissolves and the ectoderm cells pass through the same changes as did the entoderm. All of the changes in question begin at the distal end of the animal and progress basipetally.

Various external and internal conditions have been assigned as factors which induce the state of depression. Principal among them are: long continued rich feeding (Frischholz, '06, Rehm, '25); extremes of temperature (Goetsch, '22*b*, Grosz, Rehm, Frischholz); lack of oxygen or fouling of the water (Frischholz, Goetsch, Rehm, Grosz); transfer to clear fresh water (Frischholz, Goetsch); infestation with protozoan symbionts (Grosz, '25). Starvation, moderately low temperatures, and transfer to properly prepared culture water delay the onset of a depression period and lengthen the intervals between depressions (Frischholz).

My own observations on the phenomenon of depression are in accord with those of my predecessors.[1] Temperatures over 20° C., long continued rich feeding, lack of oxygen, accumulation of metabolic products and dead daphnids in the culture, and transfer to fresh clean water are conditions that tend to induce depression, often very rapidly. Lowered temperature, frequent removal of organic debris, and small replacements of the water with prepared culture water at intervals are the chief methods of delaying depression, that is, of increasing the period between depressions and of assisting recovery from depression. In spite of all care, cultures of long standing will undergo depression at intervals. The best method that I have found for bringing about recovery of depressed specimens is their transference to a culture in which hydras are flourishing. The beneficial effect of such transfer is almost incredible. Elongation of body and tentacles is often evident within an hour. Depressed specimens that have refused to feed for several days may accept food within fifteen minutes after being placed in a culture containing healthy hydras. Other less effective methods are aëration and partial replacement of the water with prepared culture water.

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[1] Except that I do not think protozoan symbionts cause depression.

In my opinion the primary cause of depression is a general lowering of the rate of physiological processes, or more briefly, a condition of senescence. According to Child ('15, p. 58) "senescence is primarily a decrease in rate of dynamic processes conditioned by the accumulation, differentiation and other associated changes of the material of the colloid substratum." The principle of indirect susceptibility (Child, '13), according to which animals in a low metabolic state are less able to adjust to slight adverse conditions than animals in a more active state, probably applies here. Depression ensues in senescent animals when they are subjected to slight unfavorable conditions such as lack of oxygen and accumulation of waste materials. In support of this view are the facts that young animals (personal observations) and regenerated animals (Goetsch, '21) are less likely to undergo depression that ordinary large animals. Evidence that depressed hydras are really in a low metabolic state will be presented later.

The observations of Trembley (1744, pp. 182–84), Welch and Loomis ('24) and Kepner and Jester ('27) leave little doubt that depression occurs in a state of nature, probably in consequence of too high temperature.

The relation between depression and the production of abnormalities is discussed later.

Irregular Budding and Colony Formation.

Ordinarily budding occurs at a definite level of the column and this fact gives rise to the impression that there exists a fixed budding zone. Such an idea is, however, erroneous, since under proper physiological conditions, buds may arise anywhere on the column except within a certain distance of the peristome[1] and probably a less distance of the foot. The occurrence of irregularly disposed buds on non-sexual specimens was noted by Trembley (1744), Laurent (1844), Hertwig ('06) and Grosz ('25). Study of these cases indicates as causes of irregular budding senescence, depression, unusual elongation of the column, and operative interference with the peristome.

Adventitious budding occurs, however, most commonly on male specimens which on being fed enter upon a period of asexual reproduction following testis formation. Such irregular budding of male specimens was noted by Trembley, Laurent, R. Hertwig, Krapfenbauer ('08) and Grosz ('25) and I shall present numerous instances of it later (see Plates IV. to VI.).

Apart from its occurrence on male specimens I have observed among thousands of hydras cultivated in the laboratory but one case of irregular budding, seen in a large *P. oligactis*. This animal (Fig. 1, Plate I.), with a column some 20 mm. in length, had six buds with a possible seventh; distally there were four buds somewhat normally arranged followed by a stalk-like differentiation of the column below which was a body region bearing two, possibly three, buds irregularly disposed, followed by the regular stalk of the specimen. Excessive length of the column probably accounts for this case of irregular budding.

The essential feature of irregular budding is that the budding region is spread over a greater length of the column than normal, particularly in the direction of the peristome. Further such budding is associated with a low metabolic state, such as senescence, sexual maturity, and depression, or with an excessively elongated column. These facts suggest that normally the budding zone occurs at a certain distance from the peristome (and probably also foot) because the peristome usually maintains a certain metabolic level. Lowering of this level permits buds to form nearer the peristome and thus to spread over a greater length of column. The same result may also be brought about by an excessive elongation of the column without any lowering of the metabolic condition of the peristome. In my observations of abnormal budding in male specimens numerous cases were observed of such a distance relationship between the peristome and the location of buds. Figs. 48 to 50, Plate IV., are typical examples. In Fig. 48 is shown a portion of a male hydra, separated by transverse fission and lacking therefore a foot, whose first bud was formed at the extreme base. Six days later, the specimen was considerably elongated and had produced a second bud markedly distal to the first one (Fig. 49). Two days later, the very elongated animal (Fig. 50) was found with a number of irregularly disposed buds, the most distal of which is still about the same distance from the peristome as was the single bud of Fig. 48. Many similar cases were observed during the study of the budding of males. It was also noted that buds may occur at the extreme proximal end when a foot is lacking but not when a foot is present. These facts suggest that the location of the budding zone on normal hydras represents a balance between the inhibiting action of peristome and basal disk on bud formation.

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[1] The name peristome is used to designate the tentacle-bearing region of Hydra.

Brief mention may be made here of so-called colony formation in hydra, due to the retention of buds far beyond the normal period of time. Hase ('09), Koch ('11), and Grosz ('25) ascribe colony formation to such conditions as senescence and depression and my own observations concur with their opinions. Any low metabolic condition permits buds to remain indefinitely upon the parent, because the extent of control of the parent peristome is then diminished and the peristome of the bud is thus able to gain control over the adjacent parent column and foot which it uses as its own. Such retained buds frequently grow out a foot laterally on the side away from their attachment to the parent and may fasten to the substratum by such feet. Numerous cases of such colonies with several retained buds and feet are illustrated in my Plates III. to V.

Abnormalities.

In addition to irregular budding and colony formation, abnormalities in hydra consist chiefly in doubling or multiplication of tentacles, and apical and basal ends. Such abnormalities occur in laboratory cultures and are also found in nature. A lowered metabolic state appears to be the primary cause of these abnormalities, excepting such as may result from regeneration after injury. The origin of the majority of them is to be sought in recovery after depression. At the start of this recovery process, duplication or multiplication of tentacles, hypostome and peristome frequently occur, apparently because regeneration begins at two or more points instead of the usual single point. On several occasions I have observed split tentacles and doubled and tripled peristomes with corresponding sets of tentacles arising in specimens recovering from depression. Figs. 23 and 24 illustrate two specimens at the beginning of regeneration after depression showing split tentacles. This relation of depression to abnormalities was previously noted by Koch ('12), Boecker ('14), Drzewina and Bohn ('16) and Grosz ('25).

There is also one other common mode of origin of duplicate apical regions. As previously discussed, buds are likely to be retained on depressed, senescent, or sexual animals. Such retained buds may grow to be of the same size as the parent or may move distally, in either case eventually producing an apparently double-headed hydra. The origin of doubled heads from retained buds by one or the other method has been recorded by Hase ('09), Koelitz ('09), Koch ('12), and Grosz ('25) and I have observed it in my cultures several times. Fig. 21 illustrates a bud, originally located at the usual budding zone, which is moving distally by fusion with the parent; Fig. 22 shows the same specimen later, simulating a double-headed hydra. Forked tentacles may also originate from a fusion as well as from a splitting process.

The occasional occurrence in nature of hydras with double or multiple apical or basal structures or other irregularities has been recorded by many writers. During the past several years I have collected annually ten to twenty thousand individuals of an undescribed species of hydra and fixed them within a few hours after collection. Abnormal specimens have been found in these collections in the ratio of about one per thousand. Figs. 2 to 20 illustrate all of the abnormal individuals occurring among twenty thousand specimens taken in the fall of 1924. Figs. 2 to 11 are cases of doubled apical ends showing various degrees of splitting (or fusion). As already suggested such duplicities probably arise either during recovery after depression or from the retention of a bud. The smaller size of one component in Figs. 2, 3, 6, and 7 suggests the latter explanation in these particular cases. Figs. 12 to 18 are forms with doubled basal ends in various stages of splitting (or possibly fusion). Such basal duplicities may arise in recovery after depression (Koch, Boecker) or probably more commonly through the formation of an accessory foot near the middle of the column, as in Fig. 12. Such a form as Fig. 12 by a splitting process in the apical direction would give rise to cases like Figs. 14, 17, 18, etc. The inequality of the two components in these cases strongly suggests the correctness of this interpretation. Fig. 19 is undoubtedly a case of a retained bud which has formed a foot in situ, while Fig. 20 is to be interpreted as a case of fusion of a bud with a parent.

FISSION.

Longitudinal and transverse fission have been reported for hydra by a number of observers: Parke ('00), Leiber ('09), Koelitz ('08*a*, '08*b*, '09), Koch ('12), Boecker ('14), Wachs ('19), Goetsch ('19), and Grosz ('25). Some of them, particularly Koelitz, regard these processes as normal modes of reproduction while others consider them as sequels of abnormal conditions. With this latter viewpoint my own observations concur. Most cases of so-called longitudinal fission are simple basipetal splittings of double-headed animals which have arisen through recovery after depression. I have observed several such "longitudinal fissions" in my cultures. The splitting process was very slow requiring several days to reach the basal disk. After having separated to the basal disk, the animals remained in this condition for a long time without further change; eventually complete separation occurred. I have also observed numerous cases of transverse fission in male "colonies," which regularly break up into smaller components by transverse constrictions.

Thus fission in hydra is not a normal method of asexual reproduction but a mode of regulation of previously existing abnormalities. From the literature it appears also that fissions may be initiated in specimens in a low metabolic state which are not otherwise abnormal.

Metabolic State.

The following tests of metabolic state in hydra were carried out by means of the direct susceptibility method of Child. This method has been reviewed so many times in papers from this laboratory that an extended consideration of its basis and application seems unnecessary here. Briefly the method consists in determining the time between exposure and death in solution of toxic substances of appropriate concentration. In general this time is roughly proportional to the metabolic state of the organism, animals in a high state of physiological activity dying faster than those in a lower state. The animals to be tested were placed in watch glasses, usually some time before the test, so that they might attach. Unattached animals exhibit increased activity of the posterior end, which should be avoided if possible. The killing agent consisted in all cases of potassium cyanide, made up in the same water as that in which the hydras were cultured, in concentrations from 1/200 to 1/700 mol., depending on the species. After the animals had attached, the water was gently removed and replaced with the cyanide solution. The watch glass was then covered with a thin plate of glass so as to exclude air, and death observed under the microscope. Only the time elapsing between exposure and death of the whole animal will be considered here as death differences along the axis were described previously (Child and Hyman, '19).

The previous observations of Child and Hyman ('19) that metabolic rate as determined by this method is greater the smaller the animal and is increased by contraction and feeding were confirmed. In *P. oligactis*, however, very little difference in susceptibility was found between animals of different sizes except that mature buds and the smallest free specimens were more susceptible than all other sizes. The latter did not differ among themselves. Some attempt was made to discover how long the metabolic increase due to food ingestion lasted but without very definite result except that the increase continues after the prey has been ejected and seems to persist for about two days. Since size, state of nutrition, and state of contraction affect susceptibility it is evident that these factors must be eliminated or equalized before comparisons by the susceptibility method can be regarded as valid.

1. *Starvation.*—It is probable that many of the observations in the literature purporting to deal with starved hydras really concern depression. The reason for this is that in starvation experiments hydras are usually isolated from the general cultures into dishes of clear fresh water. Under such circumstances, hydras almost invariably undergo depression, a condition frequently mistaken as a consequence of starvation, with which in fact it has nothing at all to do. In order to starve hydras without subjecting them to depression, it is essential to isolate them in water from the same culture in which they have been living. They then starve while remaining normal. They gradually reduce in size and become more slender and paler in color. Small specimens reduce at a much more rapid rate than do large specimens.

In the following experiments, a number of hydras of a certain size, that attained when budding begins, were isolated in a dish of culture water. At intervals during the starvation period, their susceptibility was compared with that of fed controls. Fed controls were animals of the size just specified, isolated in the same manner from the same culture two days before the susceptibility test. Thus all "fed" controls have been starved two days and are of the same size as were the starved specimens at the beginning of the starvation period. All of the data concern *P. oligactis*. The concentration of cyanide used was 1/600 mol., a concentration in which the animals remain well expanded. In these experiments, the animals were not attached.

Four days starvation: 9 starved specimens, dead, 240 to 365 minutes, 9 fed controls, 175 to 515 minutes.

Six days starvation: 6 starved specimens, 170 to 365 minutes, 5 fed controls, 430 to 530 minutes.

Seven days starvation: 10 starved animals, 80 to 370 minutes, 4 fed controls, 280 to 420 minutes.

Eight days starvation: 5 starved specimens, 65 to 290 minutes, 5 fed controls, 335 to 430 minutes.

Ten days starvation: 7 starved specimens, 150 to 450 minutes, 5 fed controls, 430 to 555 minutes.

Eleven days starvation: 11 starved animals, 30 to 225 minutes, 6 fed controls, 390 to 540 minutes.

Fifteen days starvation: 7 starved specimens, 30 to 300 minutes, 5 fed controls, 315 to 405 minutes.

Eighteen days starvation: 5 starved animals, 50 to 390 minutes, 4 fed controls, 230 to 435 minutes.

These experiments indicate that starvation raises the metabolic rate of hydra. The effect is not very noticeable until after four days of starvation. The difference between starved and fed animals seems to be greatest in moderate stages of starvation, six to eleven days, and to diminish in more prolonged starvation, fifteen to eighteen days. It was noticed that small specimens were very much more susceptible than large ones starved the same length of time.

2. *Depression.*—The earlier comparisons of depressed and normal specimens gave irregular results, the former being more resistant in some cases, but not in others. It is probable that in many cases the control specimens were not properly comparable to the depressed animals. It is in fact somewhat difficult to obtain proper controls for naturally occurring cases of depression, because generally the entire culture undergoes depression simultaneously. This entails the use of control specimens from other cultures, a questionable proceeding, since different cultures often differ physiologically. Further, depressed specimens do not feed and the factor of starvation probably enters into the result. It was finally decided that conclusive experiments could be secured only by inducing depression artificially. As already stated this can usually be accomplished by removing the specimens from the cultures and placing them in fresh clean water. It is sometimes necessary to change this water several times to keep the animals in a state of depression. The procedure then consisted in isolating a number of specimens of a certain size (size just before budding) into a finger bowl of fresh clean well water, drawn directly from a tap, and at the same time isolating others of the same size from the same culture into a finger bowl of culture water. The former undergo depression, the latter remain normal. Neither culture is fed, both being thus in the same stage of starvation. At intervals specimens were removed from both cultures and their susceptibility compared. The depressed specimens were mostly in moderately advanced depression, with short stumps of tentacles

or none and with body contracted to a short cylindrical form. The concentration of cyanide used was 1/600 mol.; in this concentration the control specimens were always well expanded while the depressed animals tended to some degree of contraction. In some experiments fed controls (starved two days) were compared simultaneously with depressed animals and starved controls. Typical results are:

Two days depression and starvation: 3 depressed, 375 to 510 minutes, 3 controls, 150 to 285 minutes.

Four days depression and starvation: 4 depressed, 415 to 540 minutes, 4 starved controls, 240 to 375 minutes, 4 fed controls (2 days starvation), 190 to 480 minutes.

Four days depression and starvation: 4 depressed (slight depression), 380 to 665 minutes, 5 starved controls, 255 to 335 minutes, 5 fed controls, 175 to 515 minutes.

Five days depression and starvation: 4 depressed, 280 to 350 minutes, 4 starved controls, 165 to 425 minutes.

Six days depression and starvation: 6 depressed, 350 to 470 minutes, 6 starved controls, 170 to 365 minutes, 5 fed controls, 430 to 530 minutes.

Seven days depression and starvation: 12 depressed, 205 to 400 minutes, 10 starved controls, 80 to 370 minutes, 4 fed controls, 280 to 420 minutes.

These experiments show that depressed specimens are generally in a lower metabolic state than normal control specimens which have been starved for the same length of time. Occasional exceptions are met. Depressed specimens are usually not less susceptible to cyanide than normal fed specimens (starved two days). It thus appears that starvation is a factor which must be taken into account. It should further be mentioned that depressed specimens are always in a state of contraction which commonly increases when they are placed in cyanide. This factor makes it difficult to gain a true idea of their metabolic state. Finally it appeared in the experiments that animals in advanced depression were at times not very much more resistant to cyanide than the controls, in fact, in some individuals, less so. This seemed to be due to the fact that in advanced depression the apical end of the specimens is already in a state of disintegration and so falls to pieces readily.

This may account for the fact, generally apparent in the experiments, that the metabolic differences between depressed and normal animals were small in distal levels and much more noticeable in proximal levels. One seems justified in concluding that depressed specimens are in a low metabolic state, which however cannot be evaluated very accurately owing to the interference of such factors as starvation, reduction in size, contraction, and preëxisting disintegration, all factors which affect susceptibility but which cannot be eliminated as they are inherent in the depressed condition.

3. *Regeneration following Depression.*—Depressed specimens may be caused to regenerate if the water is replaced by culture water. For proper comparison, starved controls should be used. One experiment was carried out on *P. oligactis,* with KNC 1/600 mol. as follows: 9 specimens, three days depression, seven days regeneration, death, 150 to 290 minutes; 7 specimens, starved 10 days, 150 to 450 minutes; 5 fed controls, two days starvation, 430 to 555 minutes. This experiment indicates that depressed specimens, which as shown above, are in a low metabolic state, attain as a result of regeneration, a metabolic rate equal to or greater than that of specimens in the same state of starvation, and much greater than that of fed specimens.[1]

4. *Sexual State.*—Whenever material was available, susceptibility tests were run on sexual as compared with non-sexual specimens. Here again it is difficult to obtain proper controls or to decide what constitutes a proper control. Sexual specimens do not feed as a rule and are therefore generally in a state of starvation, but it is usually impossible to know precisely when they last fed. If non-sexual controls are taken from the same culture as the sexual specimens, one cannot be sure that they may not be on the verge of developing gonads. On the other hand controls from other cultures are also open to criticism. The data to be presented were collected some time ago, as in the past few years, there has not been any available material; and they are probably not as carefully controlled as they should have been. In many cases it was necessary to take specimens from different cultures and the

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[1] It is worthy of note that of the nine regenerated specimens, eight budded, despite the fact that they were greatly reduced in size, while only one of the controls, starved the same length of time, produced a bud. Loss of control by the peristome thus leads to budding.

matter of feeding was not considered at all. Only specimens of the same size were compared. In some cases the species was not determined. The data available are as follows:

P. oligactis, KNC 1/400 mol.: 8 females, death 5 to 9 hours; 9 asexual specimens of same size from the same culture, 3 to 7 hours.

P. oligactis, KNC 1/400 mol.: 7 females, 5 to 9 hours; 6 non-sexual of same size from same culture, 2 to 4 hours.

Comparisons of male and female specimens of the same size but not always from the same culture, KNC 1/200 mol., species not determined: male, 115 minutes, female 340 minutes; male 70, female 340; male 170, female 365; two males 55 and 125 minutes, female 125 plus; 2 males 80 minutes, female, over 125; male 5 1/2 hours, female, over 24 hours; 2 males 345 and 500 minutes, female, over 24 hours; asexual specimen 40 minutes, 2 males 55 and 95 minutes, female 155 minutes.

These figures indicate that sexual specimens are in a lower metabolic state than non-sexual individuals and that females are decidedly lower than males. Contrary to what one might expect, the gonads are the most resistant part of sexual specimens, the ovaries being much more resistant than the testes. Unfortunately almost no data were collected on male specimens as compared with asexual individuals.

The evidence presented in this part of the paper clearly indicates that the metabolic state of *Hydra* is subject to wide variation and that such variation is definitely correlated with such conditions as age, depression, sexual maturity, and inanition. Metabolic rate may be a causal factor in the induction of at least some of these conditions.

Sexual Maturity.

Sexual maturity in hydras seems to depend in part on internal conditions and in part on external factors, such as temperature. The formation of gonads has been induced by change of temperature by several investigators: R. Hertwig ('06), Krapfenbauer ('08), Frischholz ('09), Koch ('11), Whitney ('07), Uspenskaja ('21) and Grosz ('25). But since a certain percentage of the animals remain sterile under the altered condition it must be assumed that some other factor besides temperature is involved in bringing about sexual maturity. Nussbaum's claim that starvation induces males and rich feeding females ('92, '08) has not been verified and is probably erroneous. All other observers agree that male and female gonads are induced by the same external conditions, not by opposite ones.

I have confirmed the result of several investigators that a lowering of temperature causes the development of gonads on *P. oligactis*. On January 17, 1927, a subculture from a main culture of *P. oligactis* that had been maintained in the laboratory for several months without any signs of sexual development was placed in the refrigerator (11–13° C.). On February 5, a number of specimens were observed to be developing testes. Still further depression of the temperature (to 5° C.) did not increase the number of male specimens. The culture contained about 400 hydras, of which about 30 developed testes. On March 7 another subculture was placed in the refrigerator and on March 22, signs of testis formation were noted on several specimens; by March 28, of 80 individuals in this culture, 30 possessed testes. Throughout the period occupied by these experiments and indefinitely thereafter the main culture and other subcultures kept continuously at room temperature did not show any trace of gonads. The experimental cultures were fed at frequent intervals while in the refrigerator. On return to room temperature, the induced gonads disappeared rapidly.

On the other hand, I can confirm the statements of Grosz ('25) that hydras may become sexually mature while under laboratory conditions without any apparent change of temperature. This has occurred in my cultures on several different occasions. Thus in April, 1919, a culture of *P. oligactis* which had been in the laboratory for some time developed gonads without any apparent cause; all were females. In May, 1921, cultures were started from a few specimens of *P. oligactis;* males soon appeared in these cultures although the species is not sexually mature in nature until fall. During July, there were many males in all of the subcultures of this line, many cultures containing 50 per cent. or more male specimens. Males continued to be present abundantly in these cultures for many months. During my absence from Chicago from April to July, 1922, most of the animals died, but on my return some were found in two jars. These were used as the basis of new cultures, which flourished and in which males again appeared in September, 1922, continuing throughout October, when the culture was abandoned. Another culture of *P. oligactis*, consisting of females, had a similar history. This culture started from a single specimen, collected in July, 1921. On July 11, one female was noted in the culture. Females continued to appear in it in small numbers throughout July, August, and September, 1921, and again in March, 1922. After my return in July, 1922, the cultures were restarted and females again appeared in them during September and October.

Contrariwise, cultures may be maintained for long periods of time in the laboratory and continue completely asexual. Thus for the last four years, 1923 to 1927, although cultures of hydra have been on hand almost continuously, not a single sexual specimen has appeared in them. Since many of these cultures were started from a few specimens and were continued by subculturing for many months, it is certain that the animals were of sufficient age. As already noted, the formation of gonads can be induced in a certain percentage of specimens in such cultures by subjection to low temperature (*P. oligactis*).

It is apparently widely believed by zoölogists and is current in elementary textbooks that hydra is hermaphroditic. It is now known, however, that certain species, as *P. oligactis*, are strictly diœcious. In the diœcious species, asexually produced offspring are of the same sex as their parents, as originally determined by Frischholz ('09) and since verified by others. Nussbaum ('09) and Goetsch ('22*a*) claim to have observed change of sex in hydra within a clone but their results are generally considered doubtful.

Most of the observations on sexual reproduction in hydra in the literature were made on mass cultures. It seemed to me that it would be of interest to determine the history of individual sexual specimens. With this in mind several males and females were isolated, fed, and observed daily. The remainder of the paper will be devoted to their history.

History of Isolated Females.

In October, 1925, several sexual specimens were brought in with a collection of hydras made in the lagoon in Jackson Park, Chicago. On November 18, four females were isolated from this culture into a finger bowl. They underwent depression and only one recovered. On November 25, a second female was added from the general culture and on December 22, a third. The female culture therefore came from these three females, all of which belonged to the species *P. oligactis.*

The three females were fed daily by hand on crushed *Daphnia* or *Cyclops.* Sexual specimens are frequently unable to capture prey, according to Goetsch ('22*a*), because they lack sufficient nematocysts. I cannot agree with this opinion since sexual animals when in a healthy condition can capture and swallow small daphnids without difficulty. Depression is probably the cause of the usual failure of sexual animals to feed.

The females soon began to bud in normal fashion and at the normal location. By December 22, the culture contained 8 specimens, by December 26, 14, and by the 29th, 20 animals. The original three females while continuing to bud had retained their gonads during this budding period. The buds given off were at first asexual but on January 4, 1926, all of the specimens in the culture were seen to be developing ovaries. At this time they were able to catch daphnids by themselves. By January 8 all members of the culture bore ovaries and had ceased to bud. Many eggs, unfertilized of course, were shed and could be seen lying on the bottom. The animals were no longer successful in catching prey and about January 16, hand feeding was resumed. Budding began and the young, soon after becoming free, developed ovaries. A slight depression set in about the middle of February, but recovery occurred almost at once on transferring the animals to a bowl in which hydras had been flourishing. The ovaries began to disappear and by February 27, were practically gone; budding was abundant. By March 1, rapid budding was in progress and the gonads had vanished. The animals continued to bud until the 1st of April, when for unknown causes, all of the sexual cultures were found in a state of advanced depression. The usual procedures were followed and by April 6, some recovery was attained; but on April 15 depression again set in and all of the females died.

Throughout their entire history the females were kept at laboratory temperature. The general cultures started from asexual specimens obtained from nature in the same collection as the original three females had not produced a single sexual specimen in the period in question.

These observations show: (1) that female hydras when fed bud in the usual manner; (2) that females may bud while still retaining the gonads; (3) that females may lose their gonads and become completely asexual; (4) that specimens which have been recently female and also the asexual offspring of females tend to develop ovaries in a relatively short time; (5) that the asexually produced offspring of female *P. oligactis* are of the same sex as their parents.

History of Isolated Males.

The history of the males was very much more interesting than that of the females. The behavior of budding males completely surprised me but I found on search through the literature that certain aspects of it had been seen previously by several observers, although none of them has furnished a detailed account of the phenomenon. Trembley and Laurent noted that males bud irregularly; Hertwig ('06) denied the correctness of their observations but stated that budding males possess two budding zones instead of the usual one. My observations do not agree with Hertwig's statement. Krapfenbauer ('08) described and figured the adventitious budding of male specimens (one of his figures is reproduced by Schulze, '17); and Grosz ('25) again mentions the phenomenon. None of these observers, however, appears to have followed out the eventual fate of these curious specimens. All observers except Laurent agree that the buds arise from the column of the animal, not from the testes, and with this statement my own observations accord.

The male specimens were isolated November 18, 1925, from the same culture as were the females. This culture collected in October contained a limited number of sexual specimens which so far as could be ascertained bore gonads when collected. No additional individuals became sexual in the laboratory. Seven males were isolated which underwent depression; four recovered. These were fed daily on crushed *Cyclops* but at times they and their offspring were able to catch and ingest small daphnids without assistance. The males at all times were noticeably slow and sluggish in behavior and prone to undergo slight depression, in which condition they would not accept food. During the entire period over which they were cultivated (about eighteen months), the original individuals and their offspring continued to bear testes, except for one brief interval. The buds of male specimens in most cases bear gonads before they separate from the parents and develop additional testes soon after becoming free. In many cases, testes seemed to migrate from the parent column onto the bud as has been reported by others; but most of the testes present on buds undoubtedly develop in situ.

The species of the four males cannot be stated definitely since when the experiment was begun it was supposed that all were *P. oligactis*. Later examination of offspring showed that some were *P. oligactis* and some were *Hydra stellata*, but I do not know which of the four belonged to which species. As each behaved somewhat differently, the history of each will be presented separately.

History of Male I.—On December 12, this male was found with two buds, irregularly located as shown in Fig. 25. On December 22, three buds were present, and by December 29, it had given off two of these and developed two more in the same locations (Fig. 26). The middle region of this male had a stalk-like appearance, indicated at *a* in Fig. 26 and there was evidence that a basal disk was differentiating at this region. On January 2, 1926, the specimen appeared as in Fig. 27. It was attached by a basal disk at the stalk-like region already mentioned and the old basal disk, at *f*, Fig. 27, was being absorbed. The bud marked *b* in Fig. 26 had become persistent and had gained control over the basal part of the animal. On January 4, the specimen was very much larger, had a number of buds, Fig. 28, and was attached by two basal disks, the new and the old one, *f* and *f′*, Fig. 28. The old bud *b* was still persistent. Both the original animal and *b* had normally located budding zones. On January 6, the animal divided in two by a transverse constriction, *x*, Fig. 28. This constriction occurred just below the budding zone of the larger component; this is commonly the place where such constrictions develop. The male *I* was thus divided into two parts which will be designated hereafter as *IA* and *IB,* the former being the old animal. The fate of *IA* will be followed first. It was left without a foot or stalk by the constriction. On January 6, it appeared as in Fig. 29, having three buds at its base, where buds are usually formed in such stalkness specimens. Later it gave off two buds and the remaining bud so oriented itself as to give the appearance of a biaxial animal (Fig. 30). On January 11, another bud appeared at the base of *IA;* the two buds arranged themselves at right angles to the parent column as in Fig. 31. The two buds now separated together by a constriction at the level *x,* Fig. 31, and were designated as *ID,* the old part retaining the name *IA*. *IA* subsequently developed a foot and stalk, became approximately normal, and was not followed further. *ID* remained for some time as an apparently biaxial *Hydra,* heads oriented in opposite directions as in Fig. 31. Later, January 18, the heads swung around so as to be approximately parallel, Fig. 32. The gastrovascular cavities of the two components were perfectly continuous; the specimen possessed no foot, lying free on the bottom. Later a foot developed at the bend and both specimens budded repeatedly. On February 4, Fig. 33, a double-headed bud, which subsequently became free, was noted on one of the components. On February 9, *ID,* now very large, had the appearance shown in Fig. 34. Each of the components had a definite budding zone at the normal level and a bud of each had developed a foot in situ as at *f*. Such production of basal disks by persistent buds was very common on these male specimens; such feet may or may not be fastened to the substratum. On February 10, the specimen divided by transverse constriction at the level *x,* Fig. 34. It will be noted that the plane of constriction again passed just below the budding zone of the larger component. The two parts resulting from this fission were designated *ID*1 and *ID*2 and are shown in Fig. 35 and 36, respectively. *ID*1 was left without any stalk or basal disk, while *ID*2 bore the portion which formerly connected the two specimens. *ID*1 lost the buds it had at the time of separation, Fig. 35, but developed a new bud, Fig. 37, on February 16, at its base as is usual in stalkless specimens. It will be noted that this bud again was double-headed. This bud was given off and other buds were formed at the same place and became free. On February 23, the specimen still had no basal disk, appearing as in Fig. 48. The animal then began to increase greatly in length. The bud shown in Fig. 48 became persistent, Fig. 49, March 1, and the next bud appeared proximal to it. The specimen continued to increase greatly in length and on March 3, was found with a number of irregularly disposed buds, Fig. 50. Attention has already been called to this specimen as an example of the more distal location of buds with increasing length of column.

Returning now to *ID2*, Fig. 36, we find it presenting a complicated appearance on February 16, Fig. 38. On February 18, it divided by a transverse constriction at the level *x*, Fig. 38, into two components, a larger one, still called *ID2* and a smaller one, *ID3*, shown in Fig. 39 as it appeared on February 23. It consisted of two buds which were continuous, similar to the form shown in Figs. 32 and 33. On February 27, it appeared as in Fig. 40, each component having budded. It then divided by a transverse constriction, at *x*, Fig. 40, the fission again occurring just below the budding zone of the larger component. The larger component, Fig. 42, remained in this state for some time, but eventually again split up by transverse constrictions between the bases of the components. The smaller part resulting from the fission shown in Fig. 40, later appeared as in Fig. 41, but regulated to normal by absorption of the long stalk region. The main part of *ID2* separated by the constriction shown in Fig. 38, appeared later, February 23, as in Fig. 43, having produced additional adventitious buds and having almost absorbed the basal process which it retained from the division shown in Fig. 34. On February 24, this specimen split up by two simultaneous transverse fissions, *x*, *x*, Fig. 43, into three parts, called *ID4*, *ID5*, and *ID2*, from left to right in Fig. 43. *ID4*, shown in Fig. 44, consisted of two buds connected at their bases by a narrow stalk, which subsequently parted. The history of *ID5* is shown in Figs. 45, 46, and 47. In Fig. 47, it had become nearly normal, but had given rise to a double-headed bud.

The appearance of double-headed buds several different times in the offspring of male *ID* was very interesting. The further history of such buds was followed and is much the same for all of them. Fig. 51 shows the doubled bud of Fig. 33 as it appeared on February 16, over two weeks after its separation from the parent. Each component had budded. The same specimen is shown in Fig. 52, on February 23. It had additional buds, two of which had developed feet in situ. This specimen underwent the usual fate; it divided by transverse fission at the level *x*, Fig. 52, into two components, the larger of which is illustrated in Fig. 53, as it appeared on February 25. On February 27, Fig. 54, it was larger and with additional buds, one of which was very near the basal disk. On March 1, another transverse constriction occurred, at *x* in Fig. 54, in such a way as to cut the specimen into three parts. The subsequent history of the basal region, the part below *x* in Fig. 54, is given in Figs. 55, 56 and 57. It had given off the bud which it possessed, Fig. 54, and was left with two feet, *f*, Fig. 55. It regenerated a hydranth at the free end and another proximal to a constriction, Fig. 56. The further development of the two heads is shown in Fig. 57. The specimen then divided by a constriction at *x*, Fig. 57, into a normal small *Hydra* and a larger one bearing a process on the side of the column; this process was subsequently absorbed and the specimen became normal. Figs. 65 to 67 show the history of the double-headed bud of Fig. 37. In Fig. 65, one component had budded; in Fig. 66, both components have buds; and in Fig. 67, additional buds have appeared, some with feet in situ. It will be noted that one of these buds is again double-headed.

Returning to male *IB* separated from male *I* by the constriction shown in Fig. 28, we find it on January 6, 1926, presenting the appearance given in Fig. 58. On January 8, it appeared as in Fig. 59 and on this day divided by a constriction, at *x*, Fig. 59, into two parts, *IE* and *IF*, shown in Figs. 60 and 61, respectively. *IE*, which retained the basal process, appeared on January 16 as in Fig. 62; on January 18 it constricted off the basal process, at *x*, Fig. 62. The further history of this basal process is shown in Figs. 63 and 64. After this occurrence *IE* regulated to a normal appearance. The further history of *IF* was not followed.

History of Male II. This male continued to elongate for several weeks without other change. Finally on January 4, 1926, it was found with two buds, irregularly placed, Fig. 68. On January 6, it had four buds, Fig. 69, but by January 11, it had lost three of these. Buds continued to appear irregularly on this animal and to be given off, but on January 19, Fig. 70, the largest bud present at that time became persistent and developed a foot in situ, *f*, Fig. 70. On January 27, another bud had produced a foot in situ, Fig. 71, and these two buds persisted for some time. Fig. 72 shows male *II* on February 4 with these two persistent buds, each with a foot. Finally, the more distal of the two buds was given off, but additional buds appeared at the same location, February 9, Fig. 73. February 16, Fig. 74, only the original persistent bud remained. The animal meantime was elongating greatly. On February 19, it formed an additional foot, *f′*, Fig. 75, on its column. It then began to bud again and on March 3, appeared as in Fig. 76, still retaining the persistent bud, which had itself budded, and having in addition four buds irregularly arranged. It was now much elongated. On March 8, it constricted in two at the level *x*, Fig. 76, just below the accessory foot. The two divisions, *IIA* and *IIB*, are shown in Figs. 77 and 81 respectively. *IIA* produced many buds, Fig. 78, March 25, and became large and complicated, Fig. 79, March 29, at which time it had several feet, *f*, Fig. 79. The other protuberances in Fig. 79 are young buds. Two of the feet were close together and showed a tendency to adhere to each other. On March 31, Fig. 80, the specimen was still more complex with several buds and feet. It was lost in the general depression which occurred April 1, 1926. The history of male *IIB* is given in Figs. 81 to 84. By the division shown in Fig. 76, this specimen was left with a portion of the original column, *a*, Fig. 81. This developed a foot at its anterior end, the specimen then appearing with two feet as in Figs. 82 and 83. By March 29, Fig. 84, the specimen had nearly absorbed one foot and was approximately normal.

History of Male III.—Male *III* behaved somewhat differently from the two proceding specimens. It continued to grow and elongate for nearly three months without giving rise to any buds. On January 6, it formed an accessory foot near the middle of the column, *f*, Fig. 85; and on January 8, it divided in two just proximal to this foot, at *x*, Fig. 85. The two divisions, *IIIA* and *IIIB* are shown in Figs. 86 and 88, respectively. *IIIA* absorbed the basal process and soon became approximately normal, Fig. 87, January 18. *IIIB* behaved in an unexpected manner, but one which was found to be typical for pieces of this character. It was naturally expected that it would regenerate a head at its apical end. But this apparently never happens in pieces of this kind. Instead it formed a basal disk at the apical end and thus appeared as an elongated piece with a foot at each end, Fig. 88. By January 13, it had formed an additional foot near its middle, thus having three feet, *f, f, f,* Fig. 89, all of which were fastened to the substratum. On this date, also, a small bud had appeared, *b*, Fig. 89. This bud was fed with some difficulty on very tiny specimens of *Cyclops*. It began to grow rapidly and on January 21, appeared as in Fig. 91, using its three feet in tripod fashion. February 9, it was similar in appearance, Fig. 92, and on February 24, Fig. 93, the feet appeared to be in process of absorption. By March 1, Fig. 94, it was very much larger and still possessed three feet by which it was fastened. The specimen now burst into buds, so to speak, being found on March 3 with seven buds, Fig. 95, very irregularly arranged, most of them borne on the three basal regions. On March 8, the specimen divided in two at the level *x*, Fig. 95. The distal part later absorbed its basal processes and became normal. The proximal part could not be recognized later.

History of Male IV.—This animal behaved in a fashion similar to male *III*. It increased greatly in length for many weeks without budding. Finally on January 18, it developed an acessory foot on the column and on January 28, another foot distal to this one. Fig. 96 shows it as it appeared on January 28 with three feet. On February 4, it divided in two below the middle foot, *x*, Fig. 96, the two parts *IVA* and *IVB* being shown in Figs. 97 and 98, respectively. The male *IVA*, left with two feet, finally produced a bud, February 27, opposite the distal foot, Fig. 99, but this was given off and no further buds appeared. On March 12, a third foot was formed distal to the other two, Fig. 100, and on March 17, the animal divided in two below the distal foot, at *x*, Fig. 100, into two parts, *IVA1* and *IVA2*, Figs. 101 and 102, respectively.

The further history of *IVA*1 is given in Figs. 103 to 107. It budded and also showed a great tendency to produce additional feet. Thus in Figs. 105 and 106 it has four feet in close proximity and tending to adhere to each other. It finally absorbed the long basal region and on March 31, Fig. 107, was nearly normal.

We may now follow the history of *IVB,* Fig. 98, and *IVA*2, Fig. 102. Both of these were basal pieces cut off by division and both behaved alike and in the same manner as did the similar piece *IIIB,* Fig. 88. Each regenerated a foot at the distal end, Fig. 111, *IVB,* and Fig. 108, *IVA*2. As *IVA*2 already had two feet at the time of division, Fig. 100, it was now provided with three feet, Fig. 108, one at each end and one near the middle. A bud grew out at *b,* Fig. 109, March 29, and the specimen appeared March 31 as a small *Hydra* provided with two feet, Fig. 110. *IVB* formed a foot at the distal end, Fig. 112 and then gave rise to two additional feet, Fig. 113. A bud grew out from the central portion, Fig. 114, February 23. This was provided with three feet, the fourth having apparently been absorbed. The specimen was so small that I did not succeed in feeding it.

On April 1, as already noted, both the male and female cultures were found in a state of advanced depression and could not be restored. Fortunately, however, the fate of the male specimens had been followed sufficiently to give one an idea of their general behavior. There remained the offspring of the males which had been removed as fast as they became free and placed in a separate dish. This culture, consisting of offspring of the males, escaped the depression noted and was continued for nearly a year. At first all of the specimens bore testes and continued to bear them until March, 1926, when the testes disappeared. Towards the end of April testes again developed on all of the specimens of this culture and continued to be present without intermission until March, 1927, when the culture was discontinued. Some of the offspring of this male culture had, however, inadvertently been transferred into a culture of *P. oligactis.* In July, 1926, these were wholly asexual but in August testes appeared on all of them and continued to be present as long as the culture was observed (until March, 1927).

In the chief subculture of the male line, formed from the buds of the original four males, the same curious behavior that characterized the parent specimens was repeatedly observed. Irregular budding, formation of complicated "colonies," persistence of buds, formation of accessory feet, and occurrence of transverse constrictions were prevalent in this subculture for many weeks. Finally, however, nearly all of the specimens became normal. This seemed to be due primarily to a reduction in size. The animals after April, 1926, remained of small size and budded normally, but one or two specimens exhibiting the behavior described above, could always be found in the culture.

It seems superfluous to figure and describe any more cases of the irregular behavior of male specimens, as they were similar to those already noted. An unusually curious specimen of unknown origin may be worth mentioning, Fig. 115. It probably represents a "colony" after recovery from depression, as shown by its doubled tentacles. Figs. 116 and 117 illustrate further changes in this specimen, which unfortunately was lost in the general depression of April 1, 1926. Figs. 118 and 119 furnish another illustration of a distance relation between peristome and buds. This specimen, separated by transverse constriction from *ID*4, Fig. 44, formed its first bud at the extreme base, as is common in footless animals, its second bud considerably distal to the first. Six days later, the specimen, considerably elongated, had lost the more distal bud of Fig. 118, but had formed a new bud, markedly distal to the two preceding ones, Fig. 119. Numerous cases of this kind were observed.

From a consideration of all of these observations on male specimens certain general statements appear justified. Male hydras when richly fed tend to elongate greatly and sooner or later bud or give rise to accessory feet. The buds on such elongated males are always irregularly arranged and may appear at any point over a considerable length of column. They are always, however, at some distance from the main peristome. Many of the buds of such males are set free in the usual manner but some, particularly those farthest away from the main peristome, tend to become persistent. Buds, particularly persistent ones, frequently produce feet in situ on the side opposite to their point of attachment to the parent. Continued budding of the main individual and of its persistent bud or buds results in a complex colony-like form. These colonies always break up sooner or later by one or more transverse constrictions which commonly pass just proximal to the budding zone of the main component. The main component is thus left without any foot or stalk and may remain in this state for some time, budding repeatedly and again dividing by transverse fission. It may also grow a foot and stalk and become normal. When transverse fission occurs the smaller component of the colony is usually left with a piece of column; this may be cut off, whereupon it regenerates to a small hydra, or may be absorbed. In cases where elongated males do not bud but develop accessory feet, a transverse division eventually occurs, always just proximal to one of the feet. The distal portion commonly again develops accessory feet and divides proximal to a foot or may regulate to normal by absorption of the feet. The basal region cut off below a foot does not regenerate a hydranth at its anterior end in any one of several cases observed but always forms a foot at the distal end, and may develop accessory feet along its length. Such pieces then bud somewhere between the two ends; the bud if fed grows up at right angles to the long axis of the piece, using all of the feet as its base, in tripod style. Such specimens may regulate to normal by absorption of feet or may bud and divide up by transverse constrictions. Stalkless specimens resulting from transverse fission always bud first at their extreme basal ends; but as they increase in length consequent upon feeding, the buds appear at more and more distal levels.

All of the observations plainly indicate that each peristome controls a certain length of column and that budding does not occur within the limit of control. They also indicate that the amount of control is less in these male specimens than in normal animals. Each persistent bud appears to control a certain length of the adjacent parent column, whose polarity is thereby reversed, as shown by the fact that when transverse fission occurs, this portion of the column may become the basal region of the bud in question. Each foot also appears to prevent the occurrence of a bud within a certain, rather short, distance, for buds frequently form at the extreme basal end of footless specimens while they never do so when a foot is present.

The remarkable behavior of male specimens is probably dependent on two factors—their low metabolic state and their tendency to elongate when fed. Evidence was presented earlier in the paper that sexual specimens have a lower metabolic rate than comparable non-sexual animals. This is further evidenced by their general sluggishness and slow reaction time to stimuli. In a low metabolic condition the control exercised by each peristome is diminished. Moreover, as these animals are generally much elongated, a considerable portion of the column will not be under control of any peristome and thus may bud at any point. Accessory feet also tend to appear on portions of the column not controlled by a peristome. This matter of length seems to be the more important of the two factors, since male specimens which remain of small size usually bud normally. Further females bud normally (although their metabolic rate is lower than that of males) apparently because they do not elongate when fed, so far as observed.

Finally it may be pointed out that the asexually produced offspring of male *P. oligactis* and *H. stellata* are also male and tend to form testes either before they separate from the parents or shortly after. Males specimens may become entirely asexual for short periods but display a marked tendency to develop gonads again in a short time. During all of the time that the descendants of the males remained sexual, bearing numerous testes, not a single gonad appeared in the cultures started from asexual individuals obtained in the same collection with the sexual animals. It appears that when hydras have once developed sex organs they tend to remain in the sexual state for long periods of time.

SUMMARY.

1. Directions for the cultivation of hydra are given.

2. The phenomenon of depression, in which there is a shortening and gradual loss of tentacles and column from the distal end proximally, is of common occurrence in hydras and probably represents a lowered metabolic state. It is induced by rich feeding, high temperature, sensescence, fouling of the culture water, lack of oxygen, transfer to clean fresh water. Recovery may be spontaneous or may by induced.

3. Irregular adventitious budding tends to occur in senescent, extremely elongated, depressed, and sexual specimens, that is, specimens in a low metabolic state. Under such conditions buds may be retained on the parent far beyond the normal time, giving rise to so-called colonies.

4. Abnormalities, such as forked tentacles and forked distal and basal ends arise chiefly in recovery after depression. Forked distal structures may also arise by retention of a bud.

5. Longitudinal and transverse fission are probably not normal modes of reproduction of hydra but are methods of regulation of previously existing abnormalities or occur in specimens in a low metabolic state.

6. Tests by the susceptibility method have shown that:

(*a*) Starvation beyond four days increases the metabolic rate.

(*b*) Depressed animals have a lower metabolic rate than controls starved the same length of time.

(*c*) Animals recovered from depression have an equal or higher metabolic rate than controls starved the same length of time.

(*d*) The metabolic rate of sexually mature animals is lower than that of asexual controls and that of females is lower than that of males.

7. The formation of sex organs can be induced in *Pelmatohydra oligactis* in about three weeks by lowering the temperature about ten degrees.

8. In diœcious species of hydra, the sex of asexually produced offspring is the same as that of the parent.

9. Hydras may become sexually mature in the laboratory without any apparent cause. Such sexual animals and their asexual offspring tend to remain and to become sexual.

10. Sexual specimens when fed bud while still retaining the gonads.

11. Sexual specimens when fed may lose their gonads for short periods but tend to develop them again as do also their asexually produced offspring.

12. Females when fed remain of normal size and bud normally.

13. Males when fed tend to elongate greatly and thereupon produce buds irregularly and adventitiously over a considerable length of column.

14. The buds of such male specimens tend to be retained indefinitely forming complex colony-like specimens.

15. Such retained buds commonly develop a foot in situ with which they may be fastened to the substratum.

16. Colonies formed in this manner split up eventually by one or more transverse constrictions which pass just proximal to the budding zone of the main component if any or between the bases of the components when they are equal.

17. Male specimens when fed may not bud but instead may give rise to accessory feet at one or more points along the column; such tend to divide by transverse fission just proximal to one of the feet. The proximal piece formed by such a fission regenerates a foot, not a hydranth, at its distal end; it then buds near its middle and the bud if fed grows up at right angles to the axis of the piece using the two or more feet as base.

19. Reversals of polarity due to the control of parts of the parent column by buds were frequently observed.

20. All of the facts indicate that each peristome controls a certain length of column and prevents the formation of a bud within the limits of that control. The control is diminished in senescent, depressed, and sexual specimens. Each foot also controls a short length of column and prevents the formation of a bud within that distance.

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PLATE I.

FIG. 1. Abnormally elongated specimen of Pelmatohydra oligactis, showing irregular budding; tentacles of the main hydranth omitted.

FIGS. 2 TO 11. Abnormal specimens found in nature, showing duplicature of the apical end and various stages of apparent longitudinal splitting.

FIG. 12. Abnormal specimen found in nature with split basal end.

PLATE I.

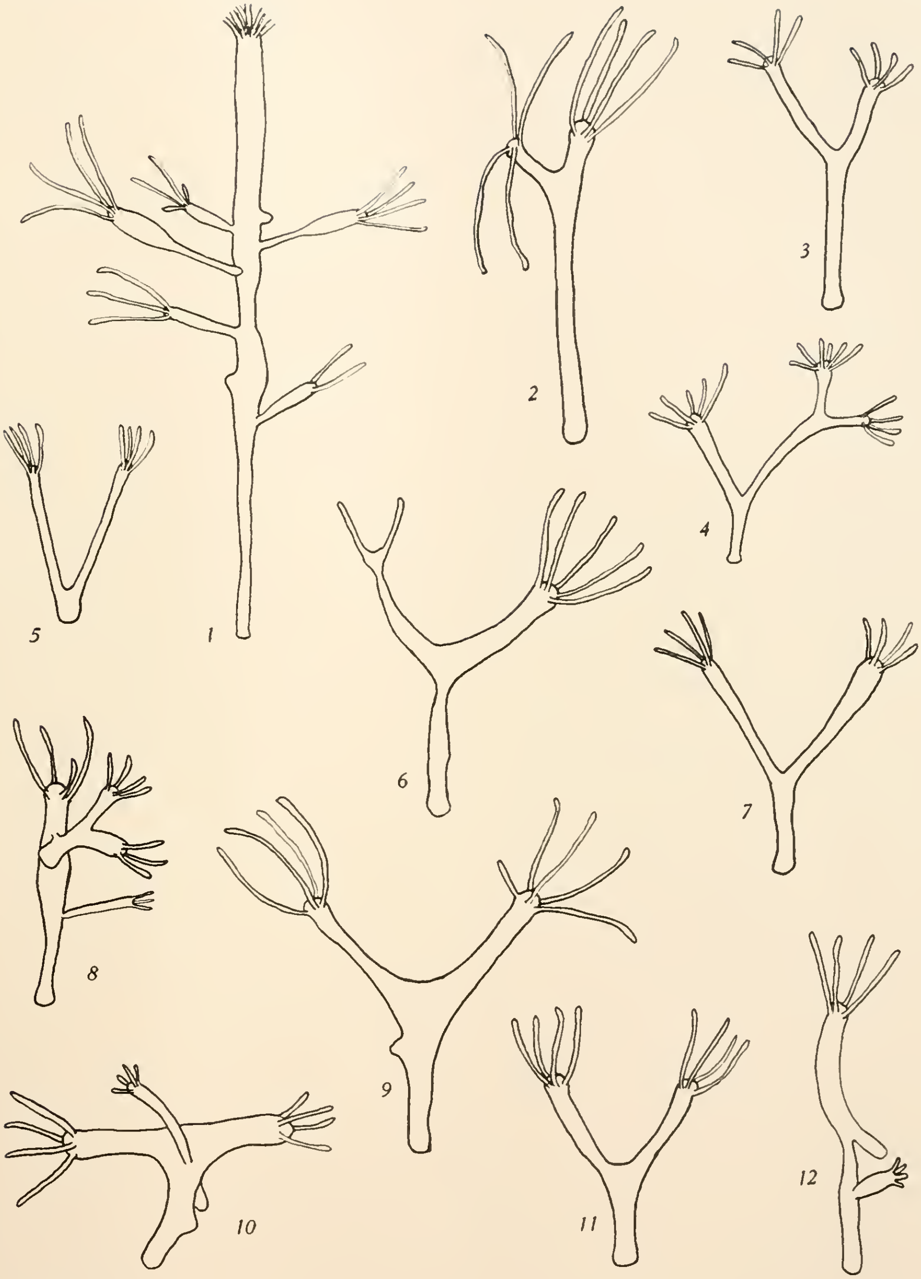
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Plate II.

Figs. 13 to 18. Abnormal specimens found in nature showing doubled basal ends, which appear to be in process of splitting in the apical direction.

Fig. 19. Specimen found in nature with a retained bud, having also a foot of its own.

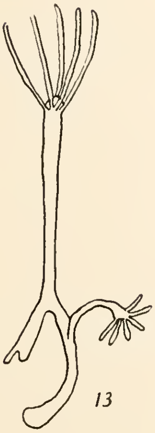
Fig. 20. Specimen found in nature, probably resulting from the partial fusion of a bud with the parent.

Fig. 21. Laboratory specimen of *P. oligactis* in which due to a slight depression, the bud *b*, originally at the normal budding zone, is moving apically by fusion with the parent.

Fig. 22. The same specimen as Fig. 21 at a later time, the bud having become equal in size to the parent, simulating a double-headed *Hydra*.

Figs. 23 and 24. Specimens at the start of recovery from depression, showing split tentacles.

PLATE II.



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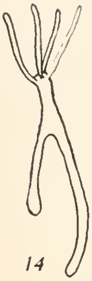

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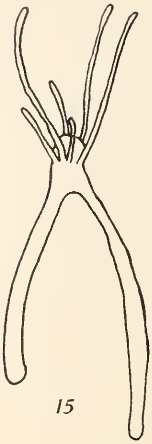

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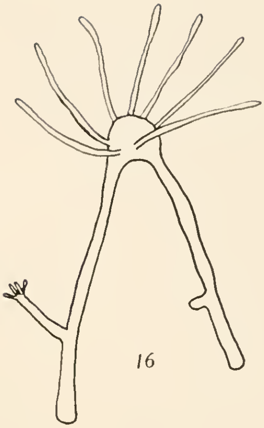

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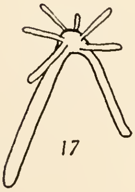

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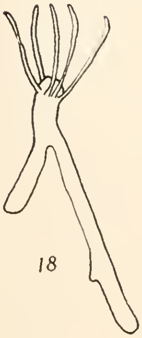

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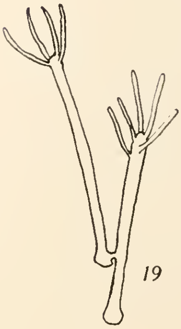

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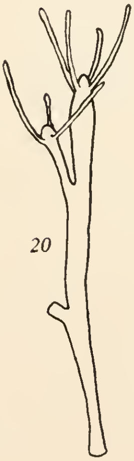

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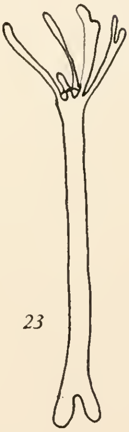

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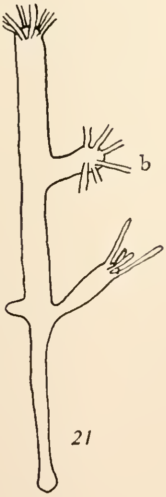

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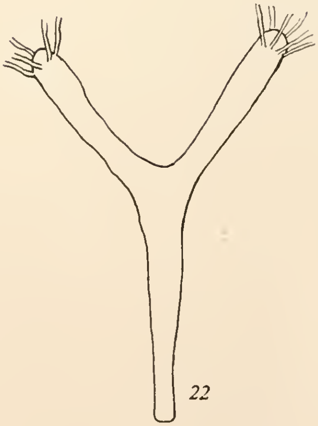

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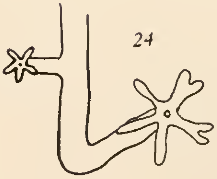

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Plate III.

Fig. 25. Male *I*, on December 12, 1925.

Fig. 26. Male *I*, on December 29, showing three buds; *b*, bud which later became persistent; *a*, region where a foot was developing.

Fig. 27. Male *I*, January 2, 1926, showing persistence of the bud *b*. *f*, the old foot, *f′*, new foot formed at the point marked *a* in Fig. 26.

Fig. 28. Male *I*, January 4, *b*, the persistent bud, *f*, the old foot, *f′*, the new foot, *x*, the level at which transverse fission occurred, on January 6.

Fig. 29. Male *IA*, distal product of the fission in Fig. 28, on January 6.

Fig. 30. Male *IA*, January 8, with one bud, oriented opposite to the parent.

Fig. 31. Male *IA*, January 11, with two buds at its base. *x*, the plane of transverse fission, January 11.

Fig. 32. Male *ID*, January 18, formed from the two buds cut off at *x* in Fig. 31.

Fig. 33. Male *ID*, February 4, one component with a double bud.

Fig. 34. Male *ID*, February 9, with four buds, two of which have a foot in situ at *f*. *x*, plane of transverse fission on February 10.

Fig. 35. Male *ID*1, right hand product of the fission shown in Fig. 34.

Fig. 36. Male *ID*2, left hand product of the fission shown in Fig. 34.

Fig. 37. Male *ID*1, February 16, with a doubled bud at its base.

Fig. 38. Male *ID*2, February 16, with three buds, each with a foot in situ. *x*, plane of transverse fission, February 18.

Fig. 39. Male *ID*3, right hand component of Fig. 38, on February 23.

Fig. 40. Male *ID*3, February 27, with several buds. *x*, plane of transverse fission.

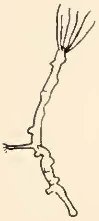
Fig. 41. Right hand part of Fig. 40 after the fission.

Fig. 42. Left hand part of Fig. 40 after the fission.

Fig. 43. Main part of male *ID*2, formed by the fission shown in Fig. 38, as it appeared February 23. *x*, *x*, planes of the two transverse constrictions which occurred February 24.

Fig. 44. Male *ID*4, the left hand part cut off by the fission shown in Fig. 43.

Figs. 45 and 46. Further history of male *ID*5, middle part cut off by the fission shown in Fig. 43.



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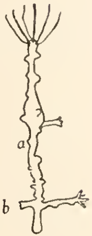

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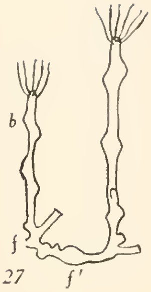

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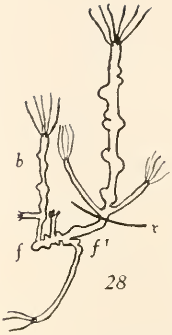

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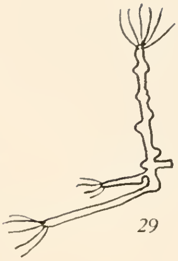

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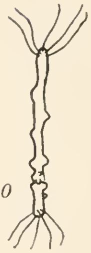

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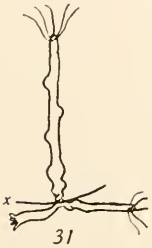

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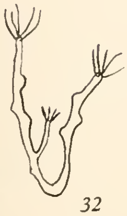

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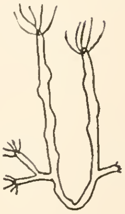

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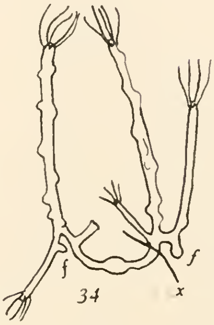

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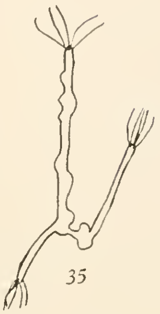

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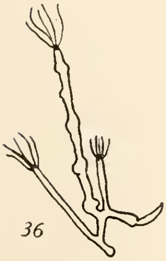

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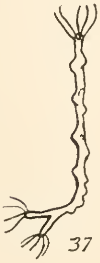

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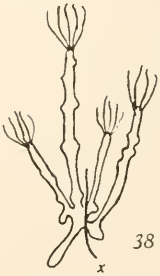

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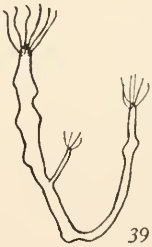

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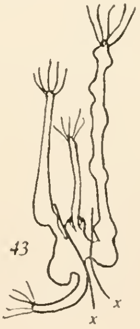

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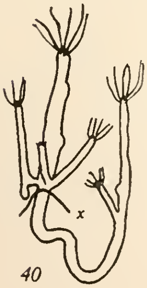

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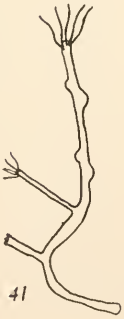

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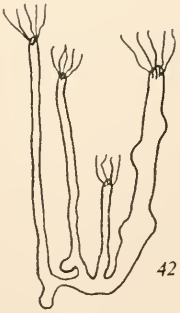

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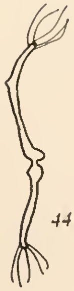

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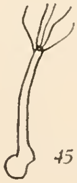

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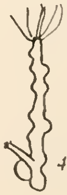

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Plate IV.

Fig. 47. Male *ID*5 later, nearly normal but with a double-headed bud.

Fig. 48. Male *ID*1, continued from Fig. 37, as it appeared on February 23, still without a stalk.

Fig. 49. Male *ID*1, March 1, with the bud of Fig. 48 persistent, and a new bud formed considerably distal to it.

Fig. 50. Male *ID*1, March 3, very elongated and with many buds, still more distally located.

Figs. 51 and 52, further history of the double-headed bud given off from the specimen shown in Fig. 33, as it appeared February 16 and February 23, respectively. *x*, Fig. 52, plane of fission.

Fig. 53. Right part of the fission in Fig. 52, February 25.

Fig. 54. Same as Fig. 53. on February 27. *x*, plane of fission, dividing specimen into three parts.

Figs. 55, 56, and 57. History of the basal piece, having two feet, *f*, *f*, separated off by the fission *x*, Fig. 54.

Fig. 58. Male *IB*, left part of the fission shown in Fig. 28, as it appeared January 6, 1926.

Fig. 59. Male *IB*, January 8. *x*, level of fission.

Fig. 60. Male *IE*, right part from the fission of Fig. 59.

Fig. 61. Male *IF*, left part from the fission of Fig. 59.

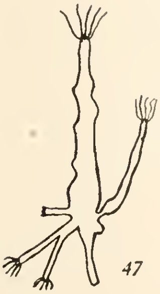
Fig. 62. *IE*, January 16. *x*, plane dividing the basal process off on January 18.

Figs. 63 and 64. Fate of the basal process separated off in Fig. 61.

Figs. 65 to 67. History of the double-headed bud given off from the specimen shown in Fig. 37.

Fig. 68. Male *II*, January 4, 1926, with two buds, irregularly placed.

Fig. 69. Male *II*, January 11, with additional buds.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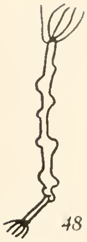
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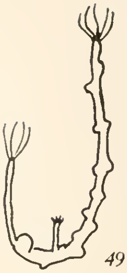
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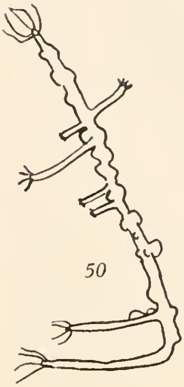
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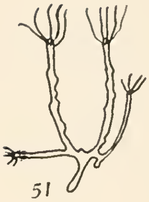
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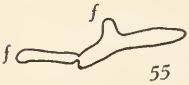

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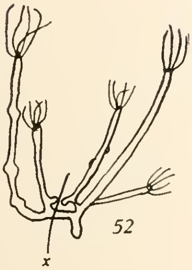

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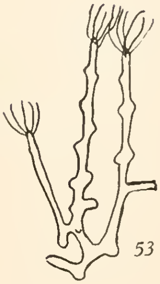
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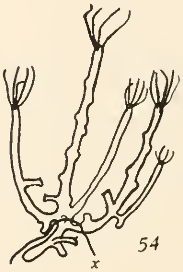

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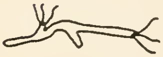
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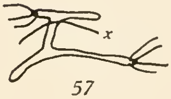

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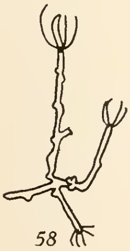
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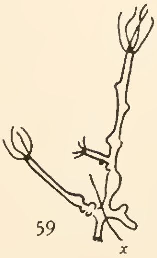

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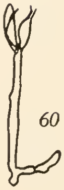
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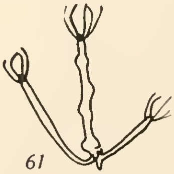
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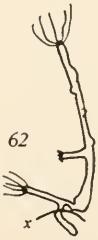

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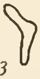
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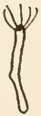
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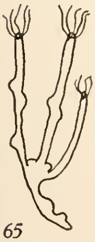
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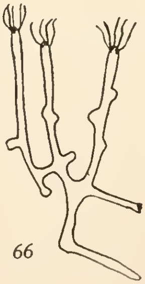
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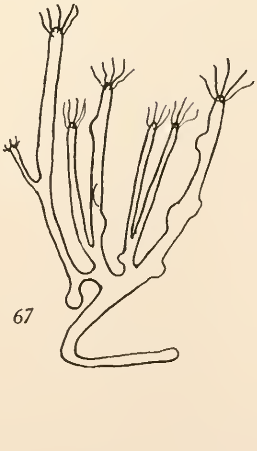
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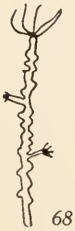
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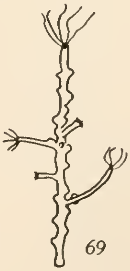
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Plate V.

Fig. 70. Male *II*, January 19, the lowermost bud persistent and with a foot, *f*.

Fig. 71. Male *II*, January 27.

Fig. 72. Male *II*, February 4, with two buds, each with a foot.

Fig. 73. Male *II*, February 9.

Fig. 74. Male *II*, February 16, with the same persistent bud.

Fig. 75. Male *II*, February 19, much elongated, with the same persistent bud and with a new foot at *f'*.

Fig. 76. Male *II*, March 3, same bud still persistent, and with additional buds. *x*, plane of the fission of March 8.

Fig. 77. Male *IIA*, distal part resulting from the fission of Fig. 76.

Fig. 78. Male *IIA*, March 25, with several buds.

Fig. 79. Male *IIA*, March 29, with several buds and feet, *f*, *f*, *f*.

Fig. 80. Male *IIA*, March 31, with many buds and feet; lost by depression.

Fig. 81. Male *IIB*, proximal part from the fission of Fig. 76. *a*, the part of the old column left on *IIB* by the fission.

Figs. 82 to 84. Further history of *IIB*. The process *a* in Fig. 81 has formed a foot at its tip, so that the specimen has two feet, both of which are functional. Fig. 83. By absorption of the basal region, the specimen became nearly normal, Fig. 84.

Fig. 85. Male *III*, January 6, with an accessory foot, *f*, near the middle of the column. *x*, plane of transverse fission, January 8.

Fig. 86. Male *IIIA*, distal part from the fission of Fig. 85.

Fig. 87. Male *IIIA*, January 18, nearly normal.

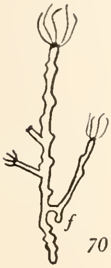
Fig. 88. Male *IIIB*, proximal part from the fission of Fig. 85.

Fig. 89. *IIIB*, January 13, with a foot at each end and one near the middle, *f*, *f*, *f*, and a bud forming at *b*.

Fig. 90. *IIIB*, January 18. It is shorter and the bud is larger.

Fig. 91. *IIIB*, January 21, the bud larger and using the three feet as its basal region.

Fig. 92. *IIIB*, February 9.



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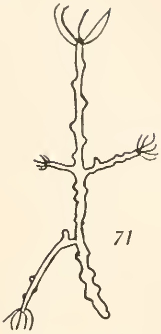

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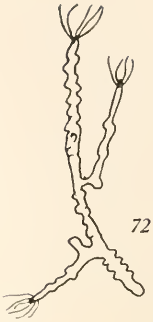

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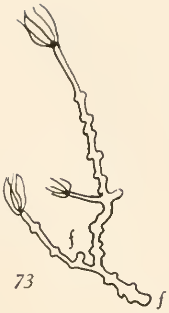

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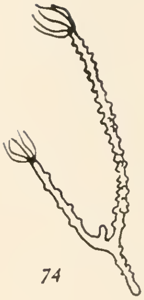

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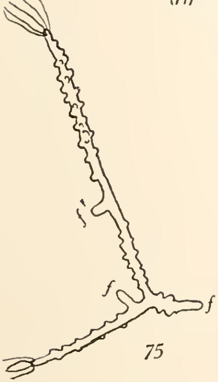

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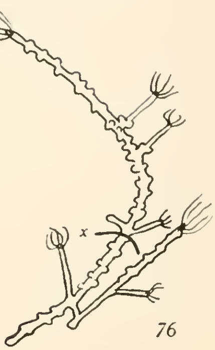

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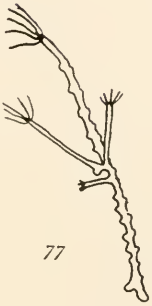

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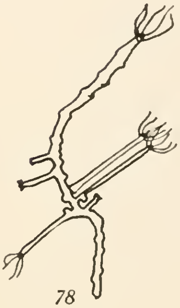

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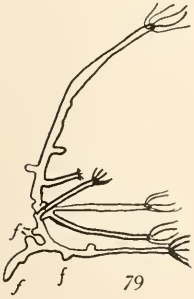

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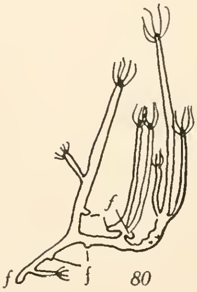

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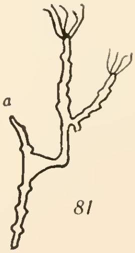

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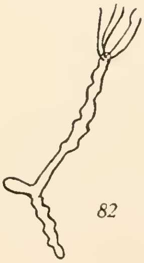

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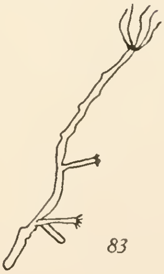

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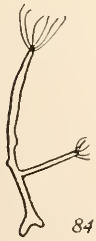

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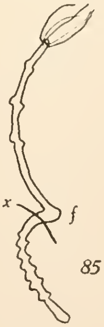

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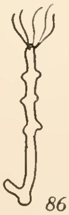

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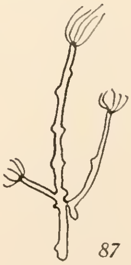

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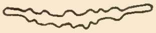

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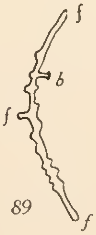

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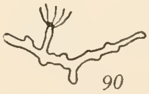

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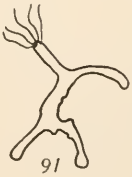

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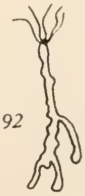

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Plate VI.

Fig. 93. *IIIB*, February 24, still with three feet.

Fig. 94. *IIIB*, March 1, much elongated.

Fig. 95. *IIIB*, March 3, with many buds. *x*, plane of fission of March 8.

Fig. 96. Male *IV*, January 28, with two accessory feet, *f*, *f*. *x*, plane of fission, February 4.

Fig. 97. Male *IVA*, distal part from the fission of Fig. 96.

Fig. 98. Male *IVB*, proximal part from the fission of Fig. 96.

Fig. 99. Male *IVA*, February 27, still with two feet and having formed a bud.

Fig. 100. Male *IVA*, March 12, with a third foot. *x*, fission plane of March 17.

Fig. 101. Male *IVA*1, distal part from the fission of Fig. 100.

Fig. 102. Male *IVA*2, proximal part from the fission of Fig. 100.

Figs. 103 to 107. Further history of *IVA*1. It formed additional feet and budded, but finally by absorption, became nearly normal, Fig. 107, March 31.

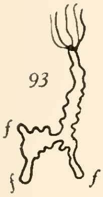
Figs. 108 to 110. Further history of male *IVA*2 (Fig. 102). It had three feet; the bud formed at *b*, Fig. 109, used these feet as base, Fig. 110.

Figs. 111 to 114. Further history of *IVB* (Fig. 98). It developed accessory feet, making four in all, Fig. 113, and a bud grew out from the middle of the specimen, which used these feet as base, Fig. 114.

Figs. 115 to 117. A peculiar specimen found in the male culture, showing regulatory changes in it.

Fig. 118. Specimen without a stalk resulting from transverse fission of *ID*4, Fig. 44. The specimen has formed two buds, one at the extreme base, the next more distal.

Fig. 119. The same specimen as Fig. 118, six days later, showing another bud formed still more distal to the preceding two. A foot has also formed at the proximal end.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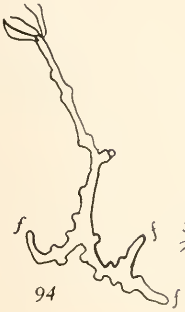
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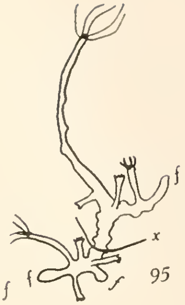
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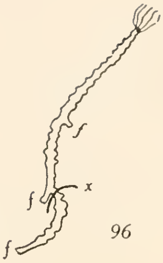
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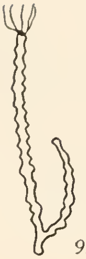
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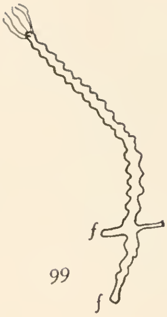
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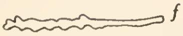
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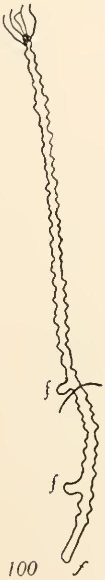
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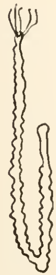
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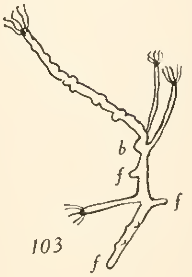
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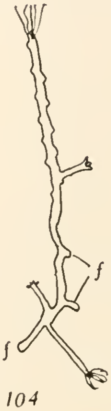
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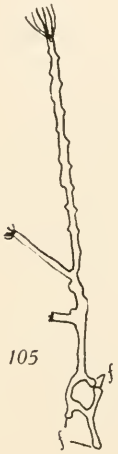
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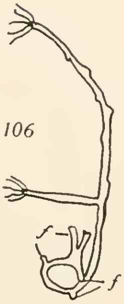
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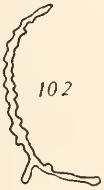
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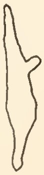
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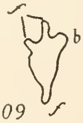
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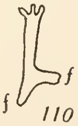
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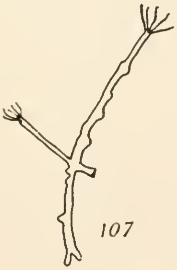
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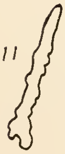
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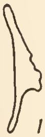
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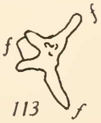
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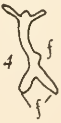
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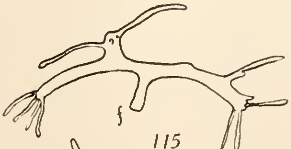
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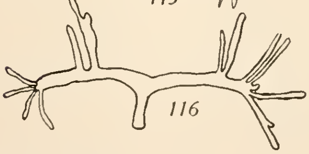
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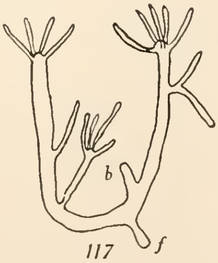
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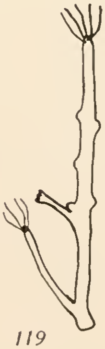
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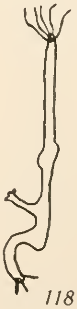
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BIOLOGICAL BULLETIN

STREAMING AND POLARITY IN *MASTIGINA HYLÆ* (FRENZEL).

ELERY R. BECKER,

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Mastigina hylæ, a member of the order Rhizomastigina, and an inhabitant of the rectum of the bullfrog tadpole, is from a strictly morphological standpoint a flagellate. The nucleus lies in the extreme anterior end, and near it are to be found the basal body which gives rise to a single, inconspicuous flagellum, and certain intracytoplasmic structures which the writer (1925) has previously described. From a physiological standpoint, however, the organism is an amœba, for the cytoplasm is continuously engaged in a process of "fountain streaming," and the single flagellum is inactive so far as can be observed. Before this flagellum was noted, the writer (1923) published a note in which he referred to this organism as "an entozoic amœba." This miscomprehension of the structure of the cell led to certain misinterpretations of the experiments which were reported in that note.

It is convenient to divide the substance in a streaming *Mastigina hylæ* into three zones: (1) the anterior zone, consisting of the nucleus, basal granule and associated structures, and the gelled cytoplastic matrix in which these structures are embedded (Fig. 1*a*); (2) a semi-fluid posterior or tail region, whose substance takes no part in the general cytoplasmic streaming (Fig. 1*c*); (3) the more extensive intermediate streaming region (Fig. 1*b*). The cell substance and ingested materials of this intermediate zone are reflected posteriad from the posterior surface of the nucleus and remaining portions of the anterior zone and anteriad from the concave anterior surface of the tail region.

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[1] The experiments reported in this paper were begun while the writer was located at Princeton, and completed at Iowa State College during the summer of 1926.

It is then the intermediate zone which is almost entirely involved in the process of streaming. We may, like Prof. John A. Ryder (1893), conceive of this streaming system as having the configuration of an elongated smoke ring, a system in which "some of itself may continuously flow through itself in order to make the progressive forward movement of itself possible." There is an axial stream which flows swiftly and continuously toward the anterior end (Fig. 1). Upon encountering the posterior surface of the nucleus and nearby region the materials in this axial stream are deflected radially to the periphery and then reflected backwards about the axial stream, forming a hollow cylinder of "ectoplasm" about it whose contents flow counter to it. The cylinder substance enters the axial stream again in the posterior region, although some part of it may have done this before the posterior region is reached. Actual timing shows that it takes from 8 1/2 to 11 5/6 seconds for a particle to make the complete circuit in healthy streaming cells. It is important to keep in mind that the nucleus is constantly at the anterior end of the long axis of the cell, where it may appear to oscillate slightly from side to side.

At any instant the living *Mastigina* has somewhat the shape of a helix of from one and one fourth to two complete coils. The coils of the helix are being constantly formed at the anterior end, while they fade out at the posterior end. The shape of the body is due to the fact that the nucleus and surrounding medium is jostled about somewhat after the fashion of the ball in the vertical jet of water which is sometimes seen in a shooting gallery. This accounts for the apparent oscillation from side to side as observed under the low power of the microscope. As a consequence the materials in the axial stream are deflected radially somewhat ununiformly, and the body is curved toward the arc of the cross-section receiving the lesser amount at any instant. Since the streaming is uninterrupted and steady, the natural resultant of the various factors is the construction of a helix. Sometimes, however, the organism is almost spatulate in shape.

The progressive movement of the organism as a whole is not usually the maximum to be expected for it often remains attached by its tail. It was found by actual timing that the maximum forward locomotion actually obtainable was about 480 microns per minute. The rate may be reduced to approximately zero, depending on how much the attachment of the uroid impedes progress.

The application of the colloid theory of protoplasm to amœboid movement is now so well recognized that it is hardly necessary to point out that the axial stream is the plasmasol (See Mast, '23, '26), the protoplasm about is the plasmagel. A thin tough elastic plasmalemma encloses the entire protoplasmic substance. If this is suddently broken, as by an abrupt jab with a glass needle, the entire protoplasmic mass except the nucleus and gelled anterior end goes into solution in the water medium. Flakes of the plasmalemma can also be seen floating about.

Here is a cell with a definite polarity. Should this polarity be ascribed to some factor inherent within the general cytoplasm? Or are the direction of streaming and consequently the cell axis maintained by virtue of some property of the nucleus, whether physical, chemical, or physico-chemical, which determines that the direction of the axial stream shall always be toward the nucleus? Or does the differential factor which determines the direction of the streaming operate in the anterior zone independently of the nucleus?

A number of individuals were cut squarely either across the middle or slightly anterior to the middle by means of a finely drawn hard-glass needle operated in a Chamber's micromanipulator. In every case the anterior fragment is able to resume the normal shape and streaming almost instantly (Fig. 3). The posterior fragment always rounds up somewhat, undergoes some fitful and uncoördinated cyclosis, extends and retracts a number of pseudopods from its cortical region, all without accomplishing any appreciable change of position (Figs. 2 and 4). The ability to carry on the gelation-solation process (the ectoplasmic-endoplasmic proces) does not exist within it. In about eight minutes it rounds up and streaming ceases completely. The anterior region continues to behave quite normally, even though it be as small as one fifth the area of the entire organism. It is not even necessary to sever the two areas completely, for the application of pressure by a needle held transversely to the large axis at any point in the intermediate zone will bring about the same result (Fig. 6).

When the pressure is released the two areas flow together and streaming is resumed.

If only the nucleus and a negligible amount of cytoplasm be amputated, the result as regards the enucleated amœba is the same. These observations decide definitely against the view that the direction of movement is determined by some factor inherent in the ultramicroscopic structure of the cytoplasm. Thus the differential factor which determines the direction of streaming is either in the nucleus or in the gelled cytoplasm at the extreme anterior tip. It cannot be held that streaming was arrested simply by removal of nuclear influence, for the writer (1926) and others have shown that cessation of streaming is not an immediate effect of the removal of the nucleus in amœbæ.

Oftentimes in unsuccessful attempts to cut off the entire anterior end (Fig. 7), the pressure applied served to dislocate the nucleus (and all the other anterior structures with it as was subsequently proved), and it commenced to travel toward the posterior end in the outer gel. A new anterior tip was temporarily established (Fig. 8). But always after a moment the streaming was arrested, a bulge appeared at the point where the nucleus had stopped, endoplasm flowed toward the nucleus, and the organism traveled away at right angles to its former course (Fig. 9). The uroid or tail piece seems also to be a permanent structure, for it again resumes its position at the posterior tip, and is not formed anew. Centrifuged specimens likewise show similar temporary disturbances of polarity, but these are more difficult to interpret. Experiments of this nature lend further support to the view that the factor that determines the direction of streaming is located somewhere in the anterior tip, for it persists in resuming its natural position at the anterior pole of the cell even though the polarity be temporarily disturbed.

But does this differential factor lie in the nucleus as the writer (1923) formerly supposed? In the summer of 1926 while attempting to cut off the anterior tip of one of these organisms by the free-hand method the nucleus was accidentally squeezed from the vacuole which it normally occupied, while the remaining anterior structures were undisturbed. The vacuole which the nucleus formerly occupied was plainly visible as a less refractile space in the anterior end. The nucleus traveled with the food particles, making time after time the circuit from axial stream to anterior end, backwards in the outer layer of gelled protoplasm to the posterior end, and then again into the axial stream. The polarity of the cell was not in the least disturbed. This makes necessary a reversal of the former conclusion of the writer that "the nucleus seems in some way to play a rôle in directing endoplasmic streaming, presumably by its ability to slightly liquefy the ectoplasmic gel, lowering the resistance at certain points to the force of the endoplasmic stream." This conclusion, as stated above, was based upon a misconception of the structure of the anterior end of the organism.

The more probable status of affairs is that it is the entire anterior tip of permanently gelled protoplasm which prevents gelation of the "endoplasm" behind it, and which by imperfect continuity with the outer gelled layer of the protoplasm of the intermediate zone creates a circular zone of weakened elasticity or lowered resistance to the internal pressure of the plasmasol When the anterior tip is amputated the remainder of the organism tends to round up. In such cases the plasmasol is imprisoned within a wall of plasmagel for the penetration of which it cannot muster enough internal pressure, thus making impossible the solation-gelatin process and the resultant streaming. If a normally streaming individual be agitated mechanically, it too rounds up, just as an enucleated fragment. This is known as the stimulated condition. But if left quietly for a moment one or several pseudopods are thrust out, but it is always the one with the nucleus in its distal tip which becomes the permanent anterior end of the streaming organism. The possession of the anterior zone is thus the *sine qua non* for normal streaming in this protozoön.

The writer has in rare instances observed in fragments of *Amœba dubia* which he had enucleated streaming strikingly similar to that in *Mastigina hylæ.* In such cases the contractile vacuole must be in the enucleated fragment and lie at the edge. It sometimes comes to occupy a position similar to that of the nucleus in *Mastigina hylæ* (Fig. 5). This type of streaming commences an instant after enucleation and may continue for from one to two minutes before the contractile vacuole bursts. The streaming is usually unbelievably rapid while it lasts. Such instances would certainly justify the assumption that polarity is determined by the physical presence in the gel of a foreign body which lowers the resistance of the peripheral zone to the internal pressure in its immediate vicinity.

CONCLUSION.

Mastigina hylæ shows typical fountain streaming, and during locomotion assumes the shape of a helix with the nucleus at the anterior end. If the organism be cut into an anterior and a posterior fragment, the anterior fragment continues to stream normally, while the posterior fragment rounds up, shows uncoördinated streaming and pseudopod formation, and soon dies. If just the extreme anterior extremity be severed from the cell, the entire protoplasmic mass behaves just as the above mentioned posterior fragment. This indicates that the factor which determines the direction of streaming, and hence polarity, is located in the anterior end. That the nucleus is not immediately responsible for polarity is indicated by the fact that polarity is not disturbed if it is dislodged from the vacuole it occupies without disturbing the remaining anterior structures. If the entire anterior end is made to travel posteriad with the outer gel, and a new anterior end formed, the original polarity is resumed by a cessation of streaming, and renewed streaming toward the nucleus.

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DESCRIPTION OF PLATE.

Fig. 1. A normally streaming *Mastigina hylæ* showing the three zones into which the body may be divided. Arrows show direction of streaming.

Figs. 2 and 3 show diagrammatically the difference in the behavior of fragments from the posterior and anterior portions of the body respectively. The latter streams normally.

Fig. 4. An enucleated fragment sending out broad pseudopods from the cortical gel (ectoplasm). The sol (or endoplasm) is confined closely within the gel.

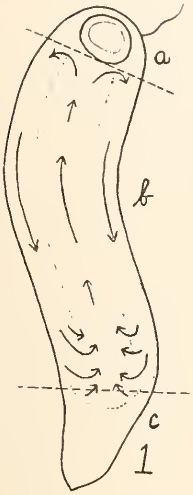
Fig. 5. An enucleated fragment of *Amœba dubia* streaming after the fashion of *Mastigina hylæ* with a contractile vacuole in the anterior tip.

Fig. 6. The effect of pressure at right angles to the longitudinal axis upon the streaming of *Mastigina hylæ*.

Fig. 7. Illustrating the method by which a break was made in the outer gel. This permitted the substance of the axial stream to rush out.

Fig. 8. The nucleus, etc., has passed to the posterior end, and a new anterior end temporarily formed.

Fig. 9. The reversal of streaming, in which the nucleus resumes its former position and the former polarity is restored.



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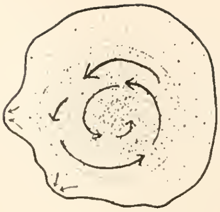

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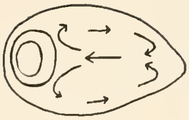

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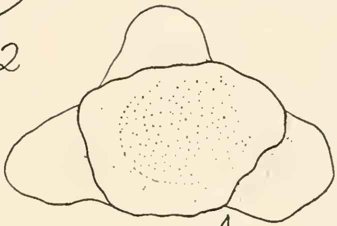

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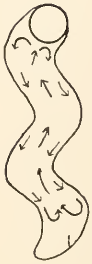

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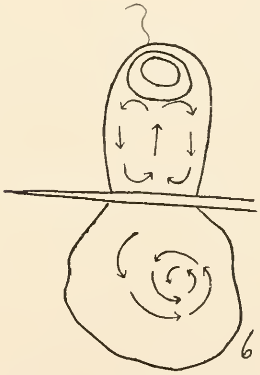

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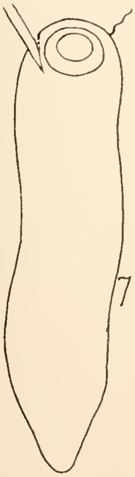

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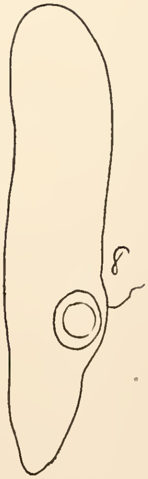

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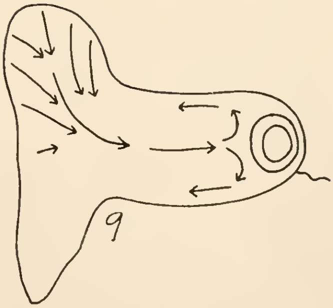

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ELERY R. BECKER.

STUDIES ON THE LIFE CYCLES OF TWO SPECIES OF FRESH-WATER MUSSELS BELONGING TO THE GENUS *ANODONTA*.[1]

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Following the pioneer work of Lefevre and Curtis (1911) in this country, much interest has centered around the investigation of the developmental cycles of the fresh-water mussels. Numerous studies have demonstrated that all members of the family Unionidæ undergo sexual development through a larval stage known as the glochidium. While Surber (1912) and others have called attention to the differences in the glochidia of many species, there are as yet many untouched possibilities of differentiating easily confused species and varieties on the basis of glochidial characters. Until quite recently it has been held that in all but two of the numerous species within this family, the glochidium must pass through a period of parasitic existence on an aquatic vertebrate before transformation to the juvenile mussel is possible. With few exceptions, fishes serve as the hosts essential for the parasitic glochidia.

It was in 1866 that Leydig made the discovery that the glochidium, after leaving the parent, completed its development as a parasite on a fish. Following that disclosure, it was believed that the parasitic life was a necessary phase in the life cycle of any fresh-water mussel. Lefevre and Curtis (1911) observed a species, *Strophitus edentulus*, which undergoes metamorphosis without parasitism, the first case cited of such a condition among the Unionidæ. This report marked an important advance in the history of the knowledge of these forms.

Three years after the discovery by Lefevre and Curtis that *Strophitus edentulus* passes through its metamorphosis in the entire absence of parasitism, Howard (1914) reported a second case of such development. He declared (1914: 353): "One of the mussels for which I found no natural infection and for which none have been reported was *Anodonta imbecillis* (Say)." Howard stated further (p. 354): "I have tested the reaction of the glochidia in the presence of fish and obtained evidence that they do not respond as other known parasitic forms."

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[1] Contributions from the Zoölogical Laboratory of the University of Illinois, No. 305.

As a result of further investigations, Howard (1911: 355) presented the account of obtaining both infection and encystment of *Anodonta imbecillis* glochidia. He also reported having obtained infection and encystment of *Strophitus edentulus*, and for this species in addition, declared actual metamorphosis to occur on fish. He concluded, therefore, that *Strophitus edentulus* glochidia may be facultatively parasitic, while for *Anodonta imbecillis*, "there is at least a persistence of the parasitic reaction when the glochidia are artificially brought in contact with fish."

As a result of investigations carried on at the University of Illinois, some observations have been made on the life histories of two species of fresh-water mussels belonging to the genus Anodonta, *Anodonta imbecillis*[1] and *Anodonta grandis*. Attention has been directed especially to a study of the glochidia and to their metamorphoses. In this connection, the occurrence of metamorphosis without parasitism has been restudied in *Anodonta imbecillis* and observations have also been made of both glochidia and juvenile mussels of *Anodonta imbecillis* and *Anodonta grandis*.

This work has been carried on under the supervision of Professor H. J. Van Cleave at the University of Illinois. The writer wishes to express to Professor Van Cleave appreciation for suggestions and guidance in this study. Further indebtedness is hereby acknowledged to R. E. Richardson for the identification of fishes used, and to F. C. Baker for aid in ascertaining the species of mussels concerned.

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[1] Baker (1927*b*) created a new genus, *Utterbackia*, with *A. imbecillis* as type. Evidences presented in this paper eliminate one of the principle characters for differentiating this genus from *Anodonta*. Under these circumstances it seems desirable to use the older name in this publication until the exact status of *Utterbackia* is determined.

METAMORPHOSIS WITHOUT PARASITISM.

Development of Strophitus edentulus.—When Lefevre and Curtis (1911) recorded their observations on the direct development of *Strophitus edentulus*, they stated that the young mussels had developed inside cords within the marsupia, reaching the stage "attained by any other unionid at the time of liberation from the host fish." The young at this stage were liberated from the marsupia by the disintegration of the cords and showed "characteristic features resembling those of *Anodonta* at the close of the parasitic period." The authors reported that they were unable to bring about the attachment of these young to fish. Since the juveniles crept about on the bottom of the dish after the manner of the young of other species in post-parasitic life, Lefevre and Curtis concluded that no subsequent parasitism was possible.

Case of Anodonta imbecillis.—Howard's discovery of *Anodonta imbecillis* passing through its metamorphosis in the absence of parasitism, brought to light a second case of this newly disclosed means of development. He secured several specimens of *Anodonta imbecillis* the latter part of November (1913). Since the species is hermaphroditic (Sterki 1898: 87), his expectations were confirmed in that all the specimens were gravid. The marsupial contents upon examination showed what at first appeared to be glochidia to be in reality "juvenile mussels with organs developed to the stage usually seen at the end of parasitism when the young mussel escapes from its host" (1914: 353–4).

In confirmation of his conclusion as to the development of *Anodonta imbecillis*, Howard stated (1914: 354): "I have tested the reaction of the glochidia in the presence of fish and obtained strong evidence that they do not respond as other known parasitic forms." He does not record, however, the species of fish used. Mature glochidia taken in March were employed which "in an exposure to fish for an hour failed to give the usual infection. A few glochidia lodged in the mouths of the fish, but no encystment could be detected. The fish showed no response." He placed especial significance upon the fact that the glochidia of *Symphonota complanata* rapidly became attached to the fish which showed considerable uneasiness in marked contrast to the behavior of fishes in the presence of *Anodonta imbecillis* glochidia.

Upon this premise, Howard thought that he had conclusive evidence that *Anodonta imbecillis* passes through its metamorphosis in the absence of parasitism.

As a result of further investigation, Howard was forced to modify his conclusions, for he reported (1914: 355) both infection by and encystment of *Anodonta imbecillis* glochidia. Howard does not record the host used in his experiments, but, since the susceptibility of fishes is significant in this connection, it is possible that in his earlier experiments he used a species entirely immune to *Anodonta imbecillis*.

Occurrence of Parasitism in *Anodonta imbecillis*.

In the present investigations, it has been found that *Anodonta imbecillis* not only discharges glochidia but that they are capable of attachment upon a fish (*Apomotis cyanellus*). The period of parasitic life is of practically the same duration as is necessary for the metamorphosis of *Anodonta grandis*.

In the course of this investigation, constant reference has been made to a comparison between conditions in *Anodonta imbecillis* and *Anodonta grandis* for the developmental cycles of these two species has been studied from a comparative point of view.

Three individuals of *Anodonta imbecillis* obtained from Lake Decatur at Decatur, Illinois, in November, 1926, were placed in a tank of running water and kept under observation. Of these, one died December 3 and was found to contain only a few living glochidia in the outer gills. The remaining two specimene wers placed in separate containers January 27. On February 1, one of these was found to have given off living glochidia which were recovered by a pipette from the bottom of the jar. During a period of twenty-four hours following, approximately 200 glochidia were discharged, which were followed by similar numbers until February 8, when the mussel was found with gaping valves. Upon examination the marsupia showed a very few of the larvæ. In the meantime, the second specimen of *Anodonta imbecillis* (2/4/27) also gave off living glochidia. After two weeks, these ceased to be discharged, the mussel living, however, until March 17, at which time an examination of the marsupia showed a few glochidial shells present but no living glochidia. It is possible that these glochidia were given off unnaturally as a result of changed environmental conditions. At any rate, the discharge was unquestionably of mature glochidia and not of young juvenile mussels.

A fairly large proportion of the glochidia discharged were active, opening and closing their valves in characteristic fashion, but in so far as these observations went were incapable of locomotion. A small green sunfish (*Apomotis cyanellus*) was subjected to a suspension of some 450 of the glochidia which had been discharged by the second specimen of *Anodonta imbecillis*. A count made after thirty minutes of exposure showed more than fifty of the larvæ attached in fairly equal distribution to the several fins of the fish. After twenty-one hours, the attached glochidia were surrounded by host tissue, encystment having been complete for at least part of the larvæ.

The infected fish was observed almost daily for the numbers of glochidia remaining. During the first few days a considerable dropping off of originally attached larvæ was noticeable. It is probable that individuals having obtained poor attachment were thus early lost.

On the eighteenth day after infection, two juveniles were recovered from the bottom of the battery jar containing the host fish. On the following days additional juveniles were secured. The following table records the number of attached larvæ and the duration of parasitism.

TABLE I.

RECORD OF THE NUMBERS OF INFECTIONS AND THE DURATION OF PARASITISM IN *Anodonta imbecillis*.

Apomotis cyanellus.	Feb.					March.										Duration of Infection.
	19	21	23	25	28	2	4	5	7	8	9	10	11	12	15	
Glochidia attached.	50	50	40	35	30	25	23	22	22	19	12	6	2	1	0	18–22 days
Juveniles recovered										2	4	3	1	1		

While Howard has reported *Anodonta imbecillis* to undergo metamorphosis without parasitism, the results of the present study do not confirm his results. This divergence in results indicates the possibility of recognizing two distinct physiological varieties of *Anodonta imbecillis.* Howard does not give a description of the species, but the characters of the glochidia presented by Surber for *Anodonta imbecillis* agree in detail with those of the species here studied. Since preserved specimens which were collected at various times of the year yielded only glochidia, showing no evidences of transformation to the juvenile stage, it is necessary to conclude that the young of *Anodonta imbecillis* forming the basis of this study are discharged as glochidia. According to the present observations, the glochidia were given off under the conditions already stated, and some actually underwent metamorphosis upon the green sunfish (*Apomotis cyanellus*).

In this connection, it may be worth mentioning that Howard presumably worked with specimens from a large stream (the Mississippi River), while the individuals forming the basis of this study were all from relatively small streams and an artificial lake formed by damming a small stream. Utterback (1916: 2) and Baker (1927a: 112) have shown that stream conditions profoundly influence the features of the adult shell in Unionidæ. Later work supplementing that given in this paper may lead to the recognition of correlation between the differences in breeding habits and environmental factors.

The Life Cycle of *Anodonta grandis.*

As mentioned earlier in this paper the observations and experiments on the development of *Anodonta imbecillis* have been paralleled by a series of similar studies on another species, *Anodonta grandis.*

A number of living specimens of *Anodonta grandis* were obtained from Lake Decatur, Decatur, Illinois, in November, 1926. A few were examined at once and found to be gravid. The marsupia were greatly distended bearing enormous numbers of living glochidia. Measurements of the shells were made from living glochidia supplemented later by measurements from permanent microscopic mounts. Table II. records the measurements taken.

TABLE II.

RECORD OF MEASUREMENTS OF GLOCHIDIA OF *Anodonta grandis.*

	Place and Date of Collection.	Extremes of Measurement.		Most Frequent Size.	
		Length.	Depth.	Length.	Depth.
Specimen No. I...	Decatur, Ill., 11/6/26	0.350 mm.-0.390 mm.	0.343 mm.-0.382 mm.	0.358 mm.	0.350 mm.
Specimen No. II..	Decatur, Ill., 11/6/26	0.350 mm.-0.398 mm.	0.358 mm.-0.390 mm.	0.366 mm.	0.358 mm.

In the literature, there is a marked discrepancy between the measurements recorded for the glochidia of *Anodonta grandis.* Thus Surber (1912: 8) states that specimens which he studied were 0.41 mm. by 0.42 mm., while Ortmann (1919: 139) found the glochidial shells to be 0.36 by 0.37 mm. The latter author called attention to the difference between his measurements and those of Surber but offered no explanation for the disagreement.

Conchologists have long recognized a subspecies of *Anodonta grandis* characteristic of the large rivers under the name of *Anodonta grandis gigantea.* The differentiation of this variety from the typical *Anodonta grandis* has been largely upon shell characters. It is known that the material examined by Ortmann (1919: 140) was from a small stream as was also the material upon which the present investigation was based. On the other hand, it is probable that Surber's material, though recorded as *Anodonta grandis,* was from the Mississippi River and consequently belonged to the variety *Anodonta grandis gigantea.* Thus it seems reasonably certain that *Anodonta grandis* and its variety *Anodonta grandis gigantea* may be differentiated with greater certainty on the basis of glochidial measurements than on shell characters of the adult.

Under conditions of the experimentation, specimens of *Anodonta grandis* kept in tanks of running water continued to retain living glochidia within the marsupia from November 22 until January 6. Following this date, some of the females bore glochidia in the anterior region of the marsupia only, while after February 10 all the females examined lacked living glochidia. According to observations of Coker, Shira, Clark, and

Howard (1921: 142), females of *Anodonta grandis* bearing glochidia have been observed in December, January, February, and March only. Thus while there is a slight difference between the observations recorded by these authors and those of the present study, the length of the gravid period is almost identical in the two instances.

Four days after the living specimens of *Anodonta grandis* had been placed in the tank, material examined from the bottom contained living glochidia. In two weeks the number of living ones was greater in a similar quantity of material, which contained also many glochidial shells. The dates of gravidity for those specimens kept under observation in running water have already been indicated. Three living specimens brought in from the field on May 7, 1927, contained no glochidia.

In order to observe the results of artificial infection with *Anodonta grandis* for a comparison with the earlier study on *Anodonta imbecillis*, several specimens of small fishes were used. One species of minnow (*Pimephales notatus*), two of darters (*Etheostoma cæruleus*, *Beleostoma nigrum*), one sucker (*Moxostoma breviceps?*), and the green sunfish (*Apomotis cyanellus*) were placed in the tank with the living *Anodonta grandis* December 6, 1926. At this time, a fairly large number of living glochidia were present in the material at the bottom of the tank. A record was kept of the numbers of glochidial attachments for three weeks. The results are shown in the following table.

TABLE III.

RECORD OF THE ARTIFICIAL INFESTATIONS BY GLOCHIDIA OF *Anodonta grandis*.

Fish Introduced into the Tank Dec. 6, 1926.	Numbers of Infestations.					
	Dec. 8.	Dec. 13.	Dec. 16.	Dec. 18.	Dec. 23.	Jan. 3.
Pimphales notatus:						
Specimen No. I	1	1	3	2	1	5
Specimen No. II	0	1	3	2	1	2
Specimen No. III	0	3	1	1	1	0
Etheostoma cæruleus	0	2	14	17	11	0
Beleostoma nigrum	0	1	4	5	2	0
Moxostoma breviceps?						
Specimen No. I	0	0	21	37	5	2
Specimen No. II	0	0	2	2	0	1
Apomotis cyanellus	1	1	1	4	5	5

The results in the case of the minnows, darters, and sucker showed considerable variation in numbers of infestations. Increased attachments were evident in all cases after the first infection, which condition was especially marked in the case of one darter (*Etheostoma cæruleus*) and one sucker (*Moxostoma breviceps?*). Particularly in the case of the darter with its habit of remaining near the bottom of the tank darting about actively at times, it is not surprising to find that fairly heavy infection resulted. In the cases of the fish first mentioned, the increased infections were shortly followed by a dropping off of the glochidia, as indicated by the reductions of the numbers carried. Examination of the fish showed parts of the fins sloughed off presenting noticeably ragged appearances especially marked in the case of the darters and suckers. The sunfish, on the contrary, did not show such a reduction in the numbers of glochidia. The fins remained intact, and examination soon showed the glochidia encysted. The sunfish was the only host encountered in these experiments which retained the parasites for any appreciable length of time. Lefevre and Curtis (1910: 103) recorded several species of Centrarchidæ as natural hosts of *Anodonta grandis*.

In order to observe the results of attachment of the glochidia to the sunfish more accurately than was possible by leaving the fish free to swim about in the tank, two small uninfected specimens were used for experimentation. Of these, one was a small green sunfish (*Apomotis cyanellus*), the same species which had appeared earlier to be subject to infection, and the other a rock bass (*Ambloplites rupestris*). A fairly large quantity of living glochidia from one of the gravid females of *Anodonta grandis* was placed with each of these fish in separate small containers. Infection was evident at once, and after fifteen minutes of exposure, each fish was placed in fresh water, again in the small containers which were now set in the tank in order to keep the water at a temperature favorable for the existence on the fish, at the same time preventing additional infection.

The two fishes kept under observation were kept in the same temperature conditions, the glochidia which they carried undergoing metamorphoses in eighteen days and in seventeen to twenty days respectively. A record of the duration of parasitism on the different fishes is given in Table IV.

Table IV.

Record of the Numbers of Infections and Duration of Parasitism of *Anodonta grandis*.

	Feb.							March.					Duration of Infection.
	16	18	19	21	23	25	28	2	4	5	7	8	
Apomotis cyanellus:													
Glochidia attached......	2	2	2	2	2	2	2	2	2	0			18 days
Juveniles recovered.....										2			
Amblo plites rupestris:													
Glochidia attached......	40	40	35	30	25	20	17	6	4	1	1	0	17–20 days
Juveniles recovered.....									2		3		

Summary.

1. *Anodonta grandis* and *Anodonta imbecillis* have been studied with reference to the glochidia and later stages in the life cycles.
2. Through experimental infestation, glochidia of *Anodonta imbecillis* have been reared under laboratory conditions through the period of transformation to the juvenile stage.
3. An examination of mature individuals collected at various times of the year yielded only glochidia, showing no evidences of transformation to the juvenile stage.
4. The claim that metamorphosis in *Anodonta imbecillis* occurs in the marsupia of the parent has not been confirmed by this investigation.
5. *Apomotis cyanellus* served as host to the glochidia of *Anodonta imbecillis.*
6. The transformation of glochidia of *Anodonta grandis* has been followed in experimental infestations using *Apomotis cyanellus* and *Amploplites rupestris* as hosts.
7. *Anodonta grandis* may be differentiated from *Anodonta grandis gigantea* on the basis of glochidial measurements.

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THE MORPHOLOGY AND PHYSIOLOGY OF THE SALAMANDER THYROID GLAND.

III. The Relation of the Number of Follicles to Development and Growth of the Thyroid in *Ambystoma maculatum*.

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Owing to the profound changes which the follicular pattern of the human thyroid gland undergoes in various pathological states, the follicular composition of the thyroid has been given careful attention by many students of the thyroid gland.

Martin Heidenhain was one of the first to examine the thyroids of various mammalian species with reference to the follicular pattern. He made the noteworthy discovery that profound differences exist between the different species of this group of animals, regarding the development and morphology of the follicular design of the thyroid. According to Heidenhain two types of follicular design must be distinguished, an association type to which belong the thyroids of the dog and cat, and a dissociation type to which belong the thyroids of the cattle and of man. In the association type the follicles which develop from the primary cell columns remain permanently in epithelial continuity, while in the dissociation type they become separated. In both types secondary "fusion" may take place. Thus in the thyroids of dog and cat secondary communication (canalization) between the adjacent follicles of an epithelial column may take place, if the quantity of secretion increases, and lead to the formation of tubuli possessing a continuous lumen. The same process may, at times, occur in man, at the stage of the primary cell columns, before splitting up into separate follicles has taken place; if it does occur, it acts inhibiting upon the

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[1] Part of the work reported in this article was carried out with the aid of the "Julius Friedenwald Fund for Medical Research" of the University of Maryland.

process of splitting up. Heidenhain made some observations also on the growth and multiplication of follicles. He states that real budding of follicles is very rare; normally each cell of the primary cell columns is destined to develop sooner or later into one individual follicle. No new follicles, beyond the original number of cells, develop. In cattle, however, budding may be observed during the period of most rapid growth, just after birth. What appear to be buds in man, are only young follicles developing out of cells which failed to separate at the time when the other cells separated, the result of an incomplete dissociation. As to a multiplication of follicles Heidenhain observed that even in case of real buds these never become separated; likewise a cleavage of larger into smaller follicles does not occur.

In 1923 Williamson and Pearse (14, 15) published a study of the follicular pattern of the human thyroid gland and reported conditions essentially different from previous observations. According to these investigators the human thyroid is composed of gland-units a certain number of which make up one thyroid lobule. These units consist of an endothelial capsule the lumen of which is part of the general lymphatic system and communicates with other units and with the general lymphatic system, by means of a stalk-like lymph capillary. Very oddly in this lymphatic capsule are contained blood vessels, the capillaries of the gland-unit, which enter the capsule through the stalk-like hilus. In addition to the blood vessels each capsule contains probably one, possibly several coiled and freely floating epithelial columns. These epithelial columns of each gland unit, being the real glandular elements of the thyroid, are completely independent of the columns of other gland units, except for the lymphatic and blood-vascular communications. The solid epithelial columns represent the primary resting condition of the gland-unit. When functioning either of two completely different states—and only one of them at a time—may develop from these solid cell-columns. In both cases a lumen appears in the center of the cell column; in one case it becomes filled with colloid, in the other case with a more fluid substance. The colloid is not a secretion; the secretion is represented by the more liquid substance which, it is asserted, leaves the lumen of the epithelial tube by way of "intracellular" tubules. Normally units in each of these three states are found together in one thyroid. But a unit which elaborates colloid cannot, at the same time, elaborate secretion. Before this is possible, it must revert to the original condition of the primary solid cell column.

So far these assertions, together with many others to be found in the publications referred to and adhered to by Williamson and Pearse in their more recent articles (16, 17) are mere statements; to support them the authors have furnished a surprisingly small number of facts. For reasons outlined specifically in a previous article we cannot subscribe to these theories at large; some of them are rather incoherent and others are based upon grossly wrong observations and interpretations. But in connection with the present article we are mostly interested to know whether in the human thyroid what impressed most observers as separate vesicles are in reality only parts of continuous tubules. It is well known and has been pointed out by Heidenhain and others that the human thyroid, in certain states, does seem to be composed of numbers of coiled, continuous tubules rather than of isolated vesicles.

Upon inspection of the thyroid of an exophthalmic goiter gland[1] we have convinced ourselves that within the larger lobules smaller areas can be distinguished, which are separated from the vicinity by finer connective-tissue strands; the follicles located within these areas suggest frequently that they might be merely parts of one larger, continuous tubule and sometimes resemble in a striking manner the condition found in *Ambystoma opacum*, illustrated in Fig. 39, Plate I. of a previous paper (11); as will be mentioned presently, in this species there really exists a condition somewhat similar to the one described by Williamson and Pearse.

Since 1922 the senior author of this article has been engaged in a study of the thyroid gland of the salamander *Ambystoma opacum;* the results of this work have been published in a number of previous articles (2 to 13). In this species of salamander the thyroid, during development, may follow either of the two types distinguished by Heidenhain (1) in mammals. But whichever type it follows, the final result is, in the main, the formation of a large and coiled tube, distended with secretion to a varying degree in different places of its course, and resembling the condition described by Williamson and Pearse for the thyroid of man. The whole gland of the marble salamander may be compared, at that stage, with one single unit of the human thyroid gland. This continuous tube forms through a process of fusion of the smaller follicles, very much as described by Heidenhain, except that in case of previous dissociation really separated follicles fuse with each other. It was found that the development of this tube-like follicle, together with the elaboration and storage of the secretion product (a mixture of unstainable and, mainly, stainable colloid) coincides with the larval period. It represents, what was called, the developmental or storage phase of a functional cycle the second phase of which is the "functional phase" or phase of colloid release, which coincides with metamorphosis and is characterized, as regards the follicular pattern, by a complete collapse of the follicular tube, caused by the sudden release of the secretion product from the lumen of the follicles. The functional phase is followed again by the storage phase which now is initiated by an enormously increased elaboration of predominantly unstainable colloid. Like in man and other mammals—and unlike the assertions made by Williams and Pearse—the two secretion products, the chromophobe and the chromophile colloid, are always elaborated in and excreted from the same cells at the same time and into the same secretion-space, although in a varying proportion.

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[1] This gland was received from Dr. A. S. Blumgarten, of the German Hospital in New York; it gives me pleasure to thank Doctor Blumgarten for his courtesy.

The important feature in the thyroid of the marble salamander and the one of especial interest in connection with the present article is the existence of a phase during which the small primary follicles fuse with each other to form a large tube, sometimes resembling a coiled tube, sometimes exhibiting a more bag-like appearance, which is distended with the secretion product of the cells. At the time when these observations were made the thyroid gland of *Ambystoma tigrinum* and of the axolotl, in addition to the thyroid of the marble salamander, were studied on sections and it was noticed that the follicular patterns of these glands differ markedly from that of the thyroid of the marble salamander; the presence of a particularly large follicle was not noticed in these glands.

It was thought desirable to make a comparative study of the follicular pattern of several different species of salamanders, to see what was essential in the follicular pattern of the thyroid in general and whether differences in the follicular pattern corresponded to certain specific physiological differences of the thyroid apparatus. As at the same time large numbers of thyroids of experimental material had to be examined, the need was felt of developing a method which would enable us to make accurate examinations of the follicular pattern without the laborious work of sectioning and as soon as possible after the removal of the organs from the animals. It was found that upon maceration with 40 per cent. nitric acid the thyroid can be taken apart completely into its individual follicles, the size and shape of the follicles can be studied and the exact number of follicles can be ascertained for each individual thyroid gland. A brief account of the results thus obtained was published in the *Anatomical Record* (Uhlenhuth, 12).

In the present article the results obtained with this method for the thyroid of the salamander *Ambystoma maculatum* only will be reported; the thyroids of other species of salamanders have been successfully investigated with this method and publication of these investigations will be made soon. It should be mentioned here that the same method can be applied very successfully also to the thyroids of warm-blooded animals (dog) and of man.

METHOD.

The animals were anesthetized with chloretone (10 cc. of an 0.5 per cent. aqueous solution of chloretone plus 40 cc. of a 30 per cent. Ringer solution) and pinned up in a tray under 30 per cent. Ringer solution. The thyroids were removed in the usual way and placed at first into a dish containing a 30 per cent. Ringer solution. From this solution they were placed directly into a 40 per cent. nitric acid (Merk's Blue Label). It is best to leave them in the acid for from 12 to 24 hours and then to transfer them into a large dish filled with tap water. The water should be changed at least once. The glands treated in this fashion should be dissected as soon as possible to avoid softening of the epithelial walls of the follicles. If this procedure is employed the connective tissue will be found disintegrated sufficiently to give way to the slightest pulling, while the follicles show great resistance and can be separated from one another without the least injury to the epithelial walls. The colloid assumes a yellowish tint, the cells are whitish. In case a part of the epithelial wall is scraped off or components belonging to one single follicle are torn asunder, the defect of the epithelium and the smooth, yellowish surface of the denuded colloid can be noticed at once.

Each thyroid was completely dissected into its individual follicles and the absolute number of follicles was ascertained (Table I., column 6). Representative follicles as to shape and size were then selected and their outlines drawn at a magnification of × 33 (Zeiss Binocular Dissection Microscope, Oc. 2, Obj. a_2).

In order to determine the relative number of the follicles, *i.e.* the number of follicles per unit of thyroid volume, it would have been necessary to determine the volume of each thyroid gland. As only in a few cases the thyroid volume was ascertained another, less accurate method was resorted to. Of each thyroid the largest optical section was drawn with the aid of a camera lucida, at a magnification of × 33, after the thyroids had been removed from the acid into the water. With the aid of a planimeter the area in cm^2. of each outline drawing was determined (Table I., column 8). By dividing the number of follicles over the area the number of follicles per cm^2. area was found for each thyroid (Table I., column 7). This method would give correct results only if the cross section of each thyroid were a circle. As some thyroids are less cylindrical than others, the values for the relative numbers of follicles as recorded here are only approximately correct. No conclusions, however, were drawn from these values beyond those suggested by the continuity and persistency of the change of this value with the progressing increase in the size of the thyroids and age of the animals.

MATERIAL.

The investigations reported in the present article were carried out on the species *Ambystoma maculatum*, in spring and summer

1925 and 1926. With the exception of the larvæ of the series CCLXII. and the sex-mature animals of the series CCLXX. the animals were reared from eggs in the laboratory. All animals were kept under the same conditions except where mention is made of the contrary. All animals of the same series are the offspring of the same female, except the animals of series CCLXII. and CCLXX. The entire material was collected by Mr. George Gray in the vicinity of Woods Hole, Mass.

The Number of Follicles.

1. Average number of follicles: The average number of follicles of the thyroid gland of the spotted salamander, as calculated from the thyroids of 88 normal animals examined since 1925, is 39.7 follicles. There is, however, a considerable variability from the average; the smallest number of the follicles in one thyroid was 5 follicles, the largest number 72 follicles (Table I., column 6).

Explanation to Table I.

The early larval stages (from No. 1 to No. 29) are arranged according to body length in mm.; the older larval stages and the metamorphosed animals are arranged according to stage, as during these periods of life the stage and not the size of the animal determines the condition of the thyroid gland.

The areas of the thyroids of the first seven animals (column 8) were drawn from the fresh gland, in all the other glands the areas were drawn after removal of the thyroids from the nitric acid into water. Comparison of the area before and after the acid treatment showed a shrinkage of the thyroid, which decreased the area by approximately 18 per cent. of the area of the untreated thyroid; therefore 18 per cent. should be subtracted from the values of the area of the first seven animals, to obtain the correct measurement.

Abbreviations.

E.pr.sl.	Eyes protrude slightly.
E.pr.d.	Eyes protrude distinctly.
E.pr.c.	Eyes protrude considerably.
Yell.N.	The even coloration of the earlier larval stages has been replaced by a yellow network on dark background.
Op.sh.	Opercular pouch, on ventral side, has started to grow to the body wall and has become shallow as compared to the previous larval stage.
G.	Gill slits.
Pieces 1 day	Cast off, for the first time, some small pieces of epithelium, 1 day before examination of thyroid.
Sh.Sk.	Shed his skin completely.
L.	Placed on land (moist filter paper).
Eats	Started taking food again.

TABLE I.

NORMAL *Ambystoma maculatum*, EXAMINED SINCE 1925.

Number.	Series.		Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Area, in cm². of Largest Optical Section of Thyroid at a Magnification of × 33.
1	CCLXXI.	3	2.5 toes; 1 balancer left.	7.5	10	—	—	0.16
2	CCLXXI.	1	2.5 toes; no balancer left.	8.0	10	—	—	0.16
3	CCLXXI.	7	3.0 toes; no balancer left.	8.4	12	—	—	0.12
4	CCLXXI.	9	3.5 toes; hind limb buds out.	9.5	21	—	—	0.33
5	CCLXXI.	10	3.5 toes; hind limb buds out.	9.6	21	—	—	0.25
6	CCLXXI.	11	4.0 toes; hind limb buds split in 2.	9.6	28	—	—	0.41
7	CCLXXI.	12	6.0 toes.	11.1	29	—	—	0.14
8	CCXLVII.	32	9.0 toes.	13.4	126	*28*	80	0.35
9	CCLXXI.	14	8.5 toes.	15.1	44	*38*	42	0.9
10	CCXLVII.	33	9.0 toes.	16.4	126	*28*	62	0.45
11	CCLIV.	10	Eyes protrude slightly.	16.5	118	*33*	—	—
12	CCXLVII.	28	9.0 toes.	17.6	74	*32*	22	1.45
13	CCXLVII.	34	Larval.	17.9	126	*32*	35	0.95
14	CCLXXIV.	1	Larval.	20.0	65	*52*	43	1.2
15	CCLXXIV.	2	Larval.	21.3	66	*28*	22	1.3
16	CCLXXI.	16	Larval.	21.4	60	*34*	16	2.2
17	CCLXXI.	19	Larval.	21.5	63	*58*	25	2.3
18	CCLXXI.	20	Larval.	22.2	63	*24*	13	1.9
19	CCLXXI.	17	Larval.	22.8	62	*31*	13	2.5
20	CCLXXI.	18	Larval.	22.8	63	*38*	16	2.4
21	CCLXXX.	1	Eyes protrude slightly.	23.4	65	*50*	14	3.6
22	CCLXXX.*a*	5	Eyes protrude slightly.	23.8	76	*42*	26	1.65
23	CCLXXX.*a*	2	Eyes protrude slightly.	24.0	70	*52*	22	2.35
24	CCLXXI.*a*	1	Eyes protrude slightly.	25.7	75	*42*	17	2.55

[1] For changes of the color pattern before and after metamorphosis see Uhlenhuth, J. Experim. Zoöl., 1917, XXIV., 237–301 (p. 243).

[2] The number of follicles could not be ascertained as this thyroid consisted of a solid and continuous mass of epithelial cells, in which only four follicles were differentiated.

TABLE I. (*continued*).

Number.	Series.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Area, in cm². of Largest Optical Section of Thyroid at a Magnification of × 33.
25	CCLXXI.*a* 2	E.pr.sl.;[1] larval color pattern.	27.9	77	*51*	16	3.1
26	CCLV. 1	E.pr.sl.	28.3	91	*37*	13	2.8
27	CCLXXIV.*a* 1	E.pr.distinctly; larval color patt.	29.2	92	*49*	14	3.4
28	CCLV. 2	E.protrude.	30.2	91	*25*	7	3.6
29	CCLXXIV.*a* 2	E.pr.distinctly; larval color patt.	32.3	92	*29*	7	4.2
30	CCLXXI.*b* 2	E.pr.sl.; yellow network.	27.2	79	*41*	14	2.9
31	CCLXXI.*a* 4	E.pr.d.; yellow network.	31.1	77	*35*	14	3.4
32	CCLXXI.*b* 3	E.pr.d.; yell.N.; Operculum shallow.	30.6	87	*56*	17	3.35
33	CCLXXI.*b* 5	E.pr.d.; yell.N.; Op. shall.	31.1	88	*72*	11	6.85
34	CCLXXIV.*a* 3	Pieces 1 day; E.pr.d.; yell.N.	33.7	96	*32*	11	2.85
35	CCLXXI.*a* 5	Pieces 1 day; E.pr.cons.; yell.N.; O.sh.	35.9	88	*35*	13	2.75
36	CCLXII. 6	Yellow N.	36.0	125	*10*	3	3.5
37	CCLXII. 4	Late larval; Eyes protrude.	37.3	125	*35*	7	4.7
38	CCLXII. 10	Late larval; Eyes protrude.	37.4	127	*30*	7	4.6
39	CCLXXI.*a* 6	E.pr.consid.; yell. spots; Op. and G. closed.	35.6	89	*65*	12	5.4
40	CCLXII. 8	Begins Sh.Sk.; Op.sh.; G. open.	37.3	126	*37*	8	4.65
41	CCLXII. 7	Begins Sh.Sk.; Op. very sh.; G. open.	42.0	126	*25*	3	7.45
42	CCLXXI.*b* 6	Just shedding; O. and G. partly open.	32.4	91	*47*	14	3.45
43	CCLXXI.*a* 3	Just shedding; Op. and G. closed.	27.8	77	*30*	10	2.95
44	CCLXII. 2	Just shed Sk.; Op. closed; G. partly op.	42.4	126	*31*	4	8.2
45	CCLXXI.*a* 12	Just shed Sk.; Op. and G. partly open.	—	91	*28*	9	3.05
46	CCLXXI.*b* 1	Just shed Sk.; Op. and G. closed.	27.1	77	*43*	17	2.5
47	CCLXII. 5	Sh.Sk. ½ hour; Op. and G. nearly closed.	40.1	125	*5*	1	4.6
48	CCLXII. 9	Sh.Sk. 3 hours.	37.0	126	*44*	8	3.5
49	CCLXXIV. 3	Sh.Sk. 0 days.	22.5	68	*48*	19	2.5

TABLE I. (*continued*).

Number.	Series.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Area, in cm². of Largest Optical Section of Thyroid at a Magnification of × 33.
50	CCLVII. 1	Sh.Sk. 0 days; Op. closed; G. open.	33.0	95	63	16	4.0
51	CCLXXI.*a* 14	Sh.Sk. 0 days; Op. and G. nearly closed.	35.2	95	24	8	3.2
52	CCLIX. 6	Land 0 days; Op. and G. closed.	30.7	120	33	11	3.0
53	CCLXXIV.*a* 5	Sh.Sk. and L. 1 day; G. closed; Op. very sh.	—	106	26	10	2.5
54	CCXXXVI. 9	Sh.Sk. and L. 1 day; Op. and G. closed.	29.9	118	2		2.4
55	CCLVI. 1	Sh.Sk. 1 day; Op. closed; G. open.	35.6	91	52	10	5.5
56	CCLVII. 5	Sh.Sk. and L. 1 day; Op. and G. closed.	27.8	123	33	10	3.35
57	CCLXII. 11	Sh.Sk. and L. 1 day; Op. and G. closed.	36.1	128	30	7	4.3
58	CCLXII. 12	Sh.Sk. and L. 1 day; Op. and G. closed.	32.4	128	37	10	4.65
59	CCLXXI.*a* 7	Sh.Sk. and L. 1 day; Op. and G. closed.	33.8	89	50	12	4.2
60	CCLXII. 1	Sh.Sk. 2 days; L. 1 day; Op. and G. closed.	40.9	120	39	5	7.2
61	CCLXXI.*a* 9	Sh.Sk. and L. 2 days; Op. and G. closed.	31.6	89	52	11	4.6
62	CCLXXI.*b* 7	Sh.Sk. and L. 2 days; Op. and G. closed.	31.7	92	71	16	4.5
63	CCLXXI.*a* 8	Sh.Sk. and L. 2 days; Op. and G. closed.	33.5	89	42	8	5.2
64	CCLXXI.*a* 13	Sh.Sk. and L. 2 days; Op. and G. closed.	34.8	92	44	6	7.9
65	CCLXII. 13	Sh.Sk. 3 days; L. 2 days; Op. and G. closed.	34.8	130	—	—	6.2
66	CCLXXI.*b* 8	Sh.Sk. and L. 3 days; Op. and G. closed.	28.6	95	67	21	3.2
67	CCLXXI.*a* 10	Sh.Sk. and L. 3 days; Op. and G. closed.	32.2	89	57	13	4.25
68	CCLXII. 3	Sh.Sk. 4 days; L. 3 days; Op. and G. closed.	39.0	124	54	—	—
69	CCLXII. 14	Sh.Sk. 4 days; L. 3 days; Op. and G. closed.	41.4	130	36	4	8.15
70	CCLXXI.*b* 4	Sh.Sk. and L. 4 days; G. absorbed.	28.8	87	32	17	2.35
71	CCLXXI.*a* 11	Sh.Sk. and L. 4 days; G. absorbed.	29.6	89	43	10	4.45
72	CCLXXIV.*a* 4	Sh.Sk. and L. 5 days; Eats 1 day.	35.4	100	47	16	2.95
73	CCLXXI.*a* 15	Sh.Sk. and L. 6 days.	33.1	96	42	7	5.7
74	CCLXXI.*a* 18	Sh.Sk. and L. 11 days.	32.2	100	63	15	4.2

TABLE I. (*continued*).

Number.	Series.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Area, in cm². of Largest Optical Section of Thyroid at a Magnification of × 33.
75	CCLXXI.*a* 19	Sh Sk. and L. 11 days. Eats 1 day.	33.1	101	*36*	15	2.45
76	CCLXXI.*a* 20	Sh.Sk. and L. 12 days. Eats 4 days.	34.9	101	*50*	9	5.4
77	CCLXXI.*a* 16	Sh.Sk. and L. 13 days.	31.1	96	*36*	12	3.05
78	CCLXXI.*a* 17	Sh.Sk. and L. 13 days.	31.6	96	*44*	10	4.45
79	CCLXXI.*a* 21	Sh.Sk. and L. 13 days.	35.2	101	*26*	6	4.5
80	CCLXXI.*a* 22	Sh.Sk. and L. 17 days. Eats 8 days.	35.3	102	*47*	14	3.6
81	CCLXXI.*a* 23	Sh.Sk. and L. 20 days.	31.8	104	*72*	25	2.9
82	CCLXXI.*a* 24	Sh.Sk. and L. 189 days; killed February.	40.5	279	*39*	10	4.1
82*a*	CCLXXI.*a* 25	Sh.Sk. and L. 197 days; killed April.	46.0	328	*36*	10	3.5
83	CCXXXIV. 12	Sh.Sk. 379 days; killed August.	51.5	474	*23*	7	6.6
84	CCXXXIV. 4	L. 403 days; killed August.	49.0	476	*24*	6	4.15
85	CCXXXIV. 13	Sh.Sk. and L. 539 days; killed February.	53.5	649	*11*	2	4.96
86	CCXXXVI. 10	L. 532 days; killed February.	42.5	650	*34*	7	4.9
87	CCXXXVI. 12	Sh.Sk. and L. 532 days; killed February.	54.2	657	*26*	8	3.3
87*a*	CCXXXIX. 10	L. 591 days; killed April.	67.0	703	*40*	3	11.2
88	CCLXX. 1	Adult female; killed May 1926.	103.5	—	*34*	3	11.2
89	CCLXX. 2	Adult female; killed May 1926.	93.0	—	*53*	5	11.0
90	CCLXX. 3	Adult female; killed May 1926.	88.0	—	*24*	3	8.6
91	CCLXX. 4	Adult female; killed May 1926.	101.3	—	*30*	2	13.6
92	CCLXX. 5	Adult male; killed August 1926.	86.2	—	*48*	3	15.5
93	CCLXX. 6	Adult male; killed August 1926.	83.5	—	*33*	3	10.8
94	CCLXX. 7	Adult female; killed Febr. 1927.	90.3	—	*52*	5	9.6
95	CCLXX. 8	Adult female; killed March 1927.	84.3	—	*59*	6	9.2
		Average			39.7	13.3	

The difference of the number of follicles is considerable not only between the thyroids of different individuals, but also between the two thyroids of the same individual (Table II.*a*).

TABLE II.*a*.

DIFFERENCES IN THE NUMBER OF FOLLICLES BETWEEN THE TWO THYROIDS OF THE SAME INDIVIDUAL.

Series Number of Animal.	Left Thyroid.		Right Thyroid.	
	Number of Follicles.	Area of Largest Optic Section of Thyroid in cm².	Number of Follicles.	Area of Largest Optic Section of Thyroid in cm².
1. CCLXXI.*a* 23.	72	2.9	50	3.2
2. CCLXXI.*a* 24	39		32	
3. CCXXXIV. 13.	11	5.0	19	6.5
4. CCXXXVI. 10.	34	4.9	37	4.2
5. CCXXXVI. 12	26	3.3	23	2.8

2. Relation of the number of follicles to the size of the gland: The differences in the number of follicles are independent of the differences in the size of the glands. The number of follicles is not determined by the size of the gland. This fact may be ascertained by direct observation. It is also evident from comparison of the number of follicles with the values of the area of the thyroids. One thyroid, for instance (No. 1 in Table II.*a*) measured 2.9 cm²., but contained 72 follicles, while the thyroid of the other side of the same animal contained only 50 follicles, although it measured 3.2 cm².

In Table II.*b* are recorded the number of follicles, the thyroid-volume [1] and the number of follicles per cm³. of thyroid-volume for both thyroids of each of two animals. It will be noticed that the left thyroid of animal No. 1 contained fewer follicles than the right one, yet the volume of the left gland was larger than that of the right gland. In animal No. 2 of Table II.*b* the larger thyroid contained more follicles, absolutely, than the right one, but relatively the number of follicles was smaller in the left one than in the right one.

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[1] The thyroid-volume was calculated in the following manner. The largest and the smallest longitudinal optical sections of each gland were outlined and the areas measured as before. The area of the largest longitudinal optical section was multiplied by the largest diameter of the smallest longitudinal optical section and the area of the smallest longitudinal optical section multiplied by the largest diameter of the largest longitudinal optical section. The two products were added and the sum was divided over 2.

TABLE II.*b*.

DIFFERENCES BETWEEN THE TWO THYROIDS OF THE SAME INDIVIDUAL IN RESPECT TO THE ABSOLUTE NUMBER OF FOLLICLES AND TO THE NUMBER OF FOLLICLES PER CM³. OF THYROID VOLUME.

Series Number of Animals.	Left Thyroid.			Right Thyroid.		
	Number of Follicles.	Volume of Thyroid in cm³.	Number of Follicles per cm³. of Thyroid Volume.	Number of Follicles.	Volume of Thyroid in cm³.	Number of Follicles per cm³. of Thyroid Volume.
1. CCXXXIV. 13	11	7.16	1.5	19	11.3	1.8
2. CCXXXVI. 12	26	4.2	6.2	23	3.2	7.1

The variability of the number of follicles of the thyroids of different animals will become evident from an inspection of Table I. There are a number of thyroids among the smallest ones (less than 3 cm². area), which are composed of over 50 follicles, while among the largest thyroids there is, for instance, one which measures 13.6 cm²., but contains only 30 follicles, and another one measuring 8.6 cm². and containing 24 follicles.

In Table II.*c* the absolute number of follicles, the thyroid-volume and the number of follicles per cm³. of thyroid-volume are recorded. As will be noticed, the variability of the number of follicles, independent of the size of the gland, is not a merely apparent one caused by an inexactness of the method of expressing the size of the thyroid by the area of the largest optical section; a similar variability is noticeable, if the size of the thyroid is expressed in volume.

3. Relation of the number of follicles to the growth of the thyroid: From an inspection of Table I. it will be obvious

that, although the variability of the number of follicles is considerable, it does not show any relation to the growth of the gland. In a general way the number of follicles remains unchanged during the entire life of the animal. This circumstance is born out most clearly by the values for the relative number of follicles (Table I., column 7). The relative number of follicles is at first high (80, 42, 62), decreases rapidly with the size of the gland and in adult animals is found to be between 2 and 5.

TABLE II.*c*.

VARIABILITY OF THE THYROID GLAND IN RESPECT TO THE ABSOLUTE NUMBER OF FOLLICLES AND TO THE NUMBER OF FOLLICLES PER CM^3. OF THYROID VOLUME.

Series Number of Animal.	Left Thyroid.			Approximate Age of Animal.
	Number of Follicles.	Thyroid Volume in cm^3.	Number of Follicles per cm^3. of Thyroid Volume.	
1. CCLXXI.*a* 24	39	5.3	7.4	1st year
2. CCLXXI.*a* 25	36	4.7	7.7	
3. CCXXXIV. 13	11	7.2	1.5	2d year
4. CCXXXVI. 10	34	7.9	4.3	
5. CCXXXVI. 12	26	4.2	6.2	
6. CCXXXIX. 10	40	21.7	1.8	3d year
7. CCLXX. 7	52	23.7	2.2	Adult
8. CCLXX. 8	59	21.3	2.8	

The thyroid gland of the spotted salamander does not grow by multiplication of its follicles, but by an increase in the size of its individual follicles.

There is, in this species, no definite relation also between the number of follicles and the development of the gland, as will be discussed presently.

SIZE AND SHAPE OF THE FOLLICLES.

1. Types of follicles: The size and shape of the follicles shows an extreme degree of variation; nevertheless a number of general types of follicles can be distinguished.

In a general way it may be stated that the shape of the follicles is the more irregular the larger the follicles are.

(*a*) The smallest follicles (primary follicles) are usually round and in most thyroids strictly spherical (Fig. 1, *a*). But in nearly every thyroid there is one or several large follicles which are fairly round, possessing no diverticula at all (Fig. 1, *b* and *c*).

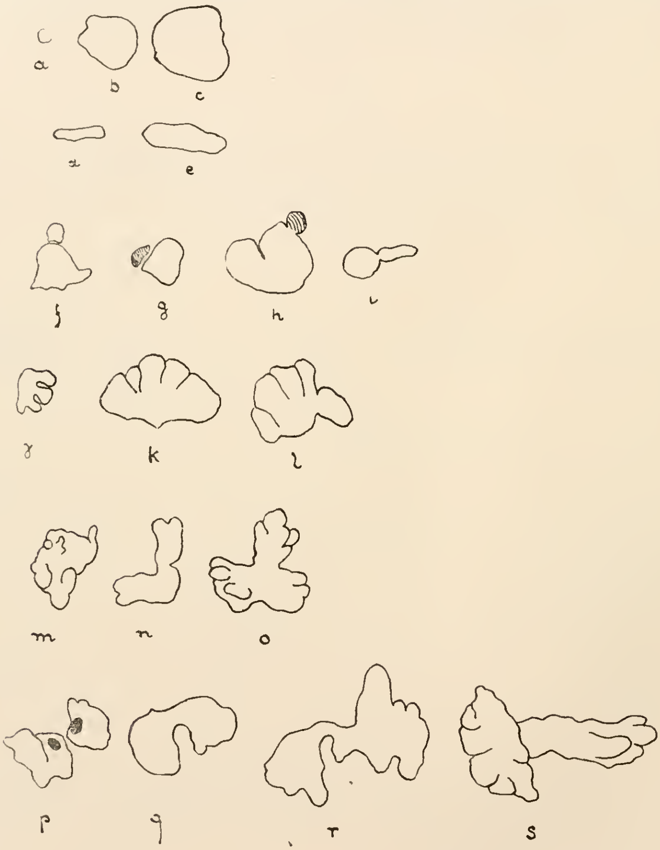

FIG. 1. Various types of follicles found in the thyroid gland of *Ambystoma maculatum*. Outlines drawn with the aid of a camera lucida, at a magnification of × 33 (Zeiss Binocular Dissecting Microscope, Oc. 2, Obj. a_2).

The cross-lined areas indicate solid follicles.

(*b*) Another kind of follicles with smooth surface are the elongate, tube-like follicles of cylindrical shape (Fig. 1, *d* and *e*).

(*c*) The simplest type of diverticulated follicle is represented

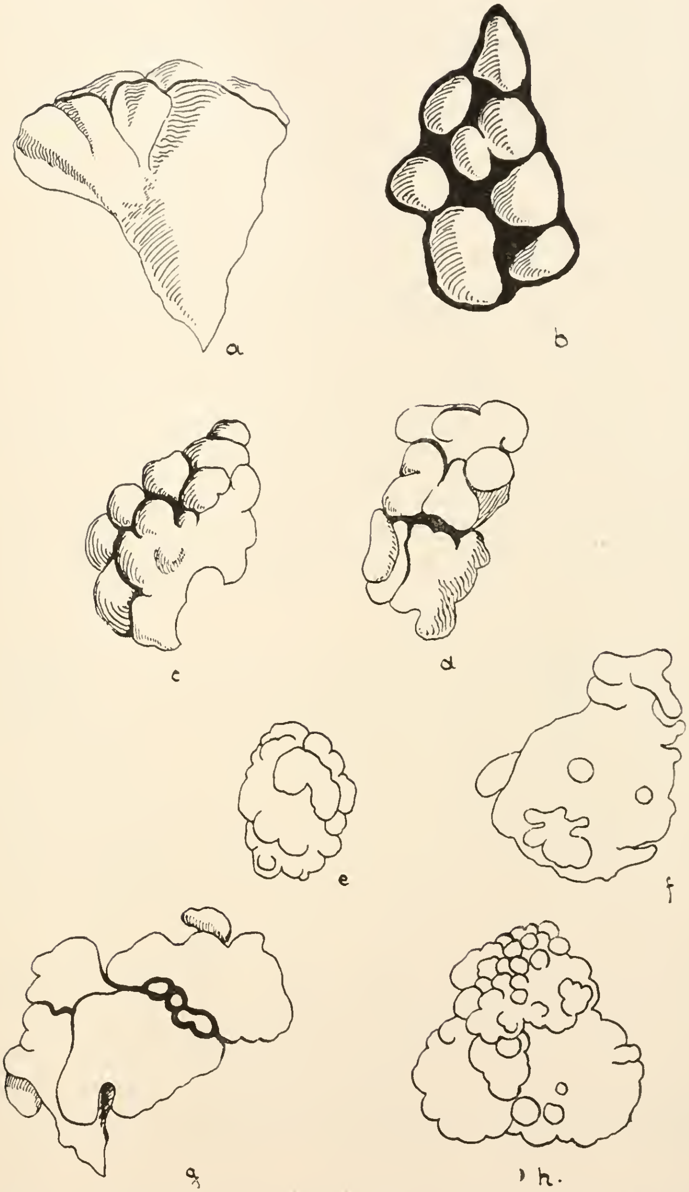

FIG. 2. Various types of follicles, found in the thyroid gland of *Ambystoma maculatum.* Drawn with the aid of a camera lucida, at a magnification of × 90 (Zeiss Binocular Dissecting Microscope, Oc. 4, Obj. *a*3). *a*, side view; *b*, view of the base of the same follicle; *c*, side view; *d*, view of the base of the same follicle. *f*, a pollicle taken from the thyroid of *Notophthalmus torosus* (CCXLVI. 2).

by fairly large, round follicles bearing on their surface one or several bud-like, spherical diverticula of the same size as the spherical primary follicles (Fig. 1, *h* and *i*). This type is rare in the spotted salamander, but is found frequently in the tiger salamander as will be shown in a subsequent paper.

(*d*) A very conspicuous type is represented by follicles of conical or pyramidal shape (Fig. 1, *j*, *k*, *l* and Fig. 2, *a* and *b*). The apex tapers into a point; from the base project two or more finger-like diverticula. In the simplest case these follicles consist of two component follicles communicating at the apex by a small hole and diverging towards the base. But usually the distance along which the component follicles are fused is much more considerable; a conical follicle results, which, at the base, is split into two finger-like processes. In many thyroids, especially noticeably so in the thyroids of sex-mature adults, a dozen or more finger-like diverticula may project from the base, while the apex is still single and pointed (Fig. 1, *k* and Fig. 2, *a* and *b*).

(*e*) Another type of follicles, especially frequent in animals metamorphosed for some time and in adults, is represented by follicles bearing so many diverticula that their shape has become completely irregular (Fig. 1, *m* and *n*, Fig. 2, *c*, *d*, *e* and *f*).[1]

(*f*) While in each of the previous types one major follicle is recognizable, bearing on its surface a varying number of smaller follicles, there is another type, the "composite follicle," which consists of two or more equally large major component follicles (Fig. 1, *o* to *s*, Fig. 2, *g* and *h*). The component follicles may communicate with one another along a considerable area of their circumference (Fig. 1, *o* and *s*, Fig. 2, *g* and *h*), or communication may be established merely by a tiny hole (Fig. 1, *p*, *q* and *r*). Particularly interesting are those follicles whose component follicles are in close apposition with one another along a considerable portion of their entire circumference, yet communicate only by a very small hole (Fig. 1, *p*) or by a short stalk. Each of the component follicles may possess a relatively smooth surface or may bear, in its turn, numerous smaller diverticula on its surface. In the latter case extreme irregularity and complexity of the follicle results.

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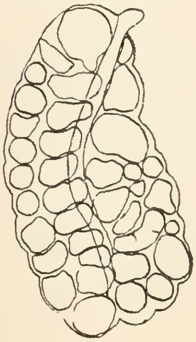
[1] *f* has been taken from the thyroid of *Notophthalmus torosus*, but is representative for many follicles of the spotted salamander.

(*g*) The smaller follicles of each type may be without a lumen, consisting of a solid mass of cells. Solid follicles are found more often among the spherical primary follicles than among the other types. In the large follicles one or several diverticula may be found solid (Fig. 1, *h*).

(*h*) Not infrequent are flat follicles; they still contain some colloid, but the lumen is very small, slit-like.

2. General arrangement of the follicles: Sometimes no definite design can be found in the arrangement of the follicles. But in most thyroids the follicles exhibit very definite relations.

The follicles are frequently arranged in perfect rows which follow the course of the main vessel (external jugular vein); the original columnar arrangement which the thyroid shows before the differentiation into follicles may be preserved thus in the thyroid of the adult (Fig. 3).



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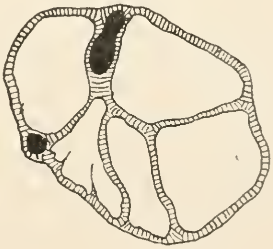

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FIG. 3. Diagram of an entire thyroid gland (Zeiss Binocular Dissecting Microscope, Oc. 2, Obj. a_2; magnified × 33), showing the arrangement of the follicles in rows parallel to the external jugular vein.

FIG. 4. Diagram of a cross section through a thyroid gland (Zeiss Binocular Dissecting Microscope, Oc. 4, Obj. a_3; magnified × 90), showing the arrangement of the follicles. White, follicles; cross lined, connective tissue; black, large veins.

The follicles are frequently so arranged, within the cross-section of the organ, that each follicle not only borders, with its central extremity, on one of the large vessels, but also comes to lie, with its peripheral surface, within the periphery of the whole organ (Fig. 4).

At each (caudate and craniate) end of the thyroid is usually one particularly large, fairly round follicle. The small spherical follicles—which in *A. maculatum* are always scarce—are usually located in the periphery of the organ (in *A. tigrinum* some thyroids contain as many as 25 per cent. extremely small follicles; here they are often crowded together into nests surrounded on all sides by larger follicles).

Characteristic is the location of the large, finger-like follicles. The apex of these follicles is always located in the center of the cross-section of the gland (Fig. 4). The finger-like diverticula point towards the periphery and their free ends come to lie, with their entire surface, within the periphery of the gland.

3. Relation of size and shape of follicles to growth and development of the thyroid: While the number of follicles bears no relation to either growth or development of the thyroid, the size and shape of the follicles is distinctly correlated to the size of the gland and, therefore, indirectly to the developmental stage of the gland.

The development of the thyroid of the spotted salamander, during the very early stages, up to the completion of the limb development, is very similar to the development of the thyroid in the marble salamander, as described in a previous paper (11). If the entire organ is removed and examined in fresh condition under the microscope, it will be seen that it consists, at first, of a solid mass of cells, devoid of any orderly arrangement of the cells. Soon this mass of cells assumes a columnar shape. The column is at first a continuous mass of epithelial cells. Gradually it becomes differentiated into small spherical groups of epithelial cells and finally each of these groups develops a lumen in its center, and differentiates into a primary follicle. At that stage maceration in nitric acid was attempted, but was unsuccessful, in as much as separation of the primary follicles was impossible. Microscopical examination shows that the connective tissue has not yet invaded the epithelial anlage; the follicles are not yet separated by connective tissue, but are in perfect epithelial continuity with each other. The youngest animal in which isolation of the follicles was partly successful was animal No. 9 in Table I., a larva possessing nearly the complete number of toes. The right thyroid of this animal was isolated into 38 portions. Of these 29 were recognized to be small individual spherical globules; although, as microscopical examination of the left thyroid showed, these globules contained a distinct lumen, the contents must have been different from the colloid of later stages, as it did not turn yellow in the nitric acid. Eight other portions of the same thyroid were of irregular shape and consisted probably partly of primary follicles and partly of solid cell-groups, all of which were connected directly by epithelial tissue. The last portion was quite large constituting a considerable part of the entire organ and being partly columnar in shape, representing in its entirety a solid mass of epithelial cells (Fig. 5, *A*).

The youngest larvæ in which complete isolation of the follicles is possible measure about 20 mm. body-length (No. 14 in Table I.) and are in the possession of completely developed toes. In the thyroids of these animals only a small number of spherical primary follicles is present (about 10 to 15 per cent.), the other follicles vary in size up to five times the size of a primary follicle and are slightly irregular in outline (Fig. 5, *B*).

By the time metamorphosis approaches (Fig. 5, *C*) the smallest follicles are still of the size of primary follicles, they are almost invariably spherical or at least roundish and their relative number is unchanged (10 to 15 per cent.). The largest follicles have not only increased considerably in size, but have become irregular in shape; among them may be represented all the types enumerated previously. Between these two extreme types of follicles transitional forms are found.

About at the time of the first skin shedding flat follicles containing almost no colloid and corresponding to the collapsed follicles found regularly in *A. opacum* after the first skin shedding are conspicuous; an exact study of that condition has not been possible with the maceration method.

The follicular pattern as found in the late larval stages remains almost unchanged during the entire life of the individual. In the adult animal the largest follicles are still larger and more irregular. Among the smallest follicles there may be found, in the first and second year, still one or several small, spherical

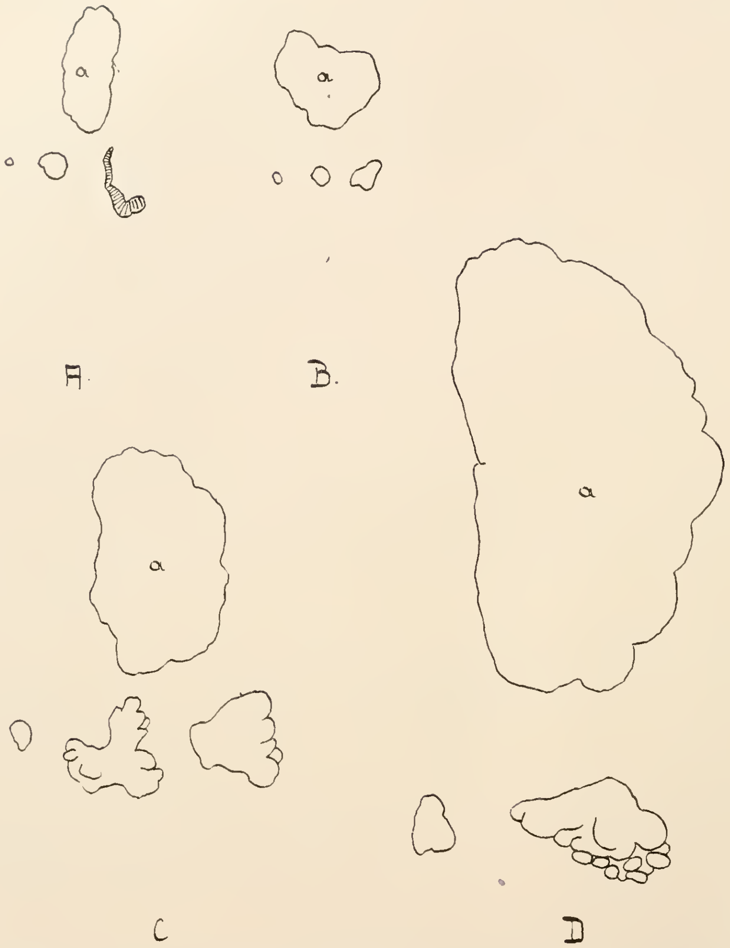

FIG. 5. Four thyroid glands of different developmental stages of *A. maculatum*, showing the changes of the follicles in size and shape. *a*, outlines of the largest optical section of each thyroid gland; below the outlines of representative follicles of each gland. Cross-lined areas indicate a solid cell mass. Outlines drawn with the aid of a camera lucida, at a magnification of × 33 (Zeiss Binocular Microscope, Oc. 2, Obj. a_2).

A. Thyroid of a larval animal (No. 9 in Table I.) of 44 days of age and 15.1 mm. body length.

B. Thyroid of a larval animal (No. 14 in Table I.) of 65 days of age and 20.0 mm. body length.

C. Thyroid of a larval animal (No. 29 in Table I.) of 92 days of age and 32.3 mm. body length, approaching metamorphosis.

D. Thyroid of an adult, sex-mature male (No. 92 in Table I.) of 86.2 mm. body length (age unknown).

or at least roundish follicles of the size of a primary follicle. In the sex-mature adults, however, the smallest follicles are larger than the primary follicles and may be five or more times as large as a primary follicle (Fig. 5, *D*).

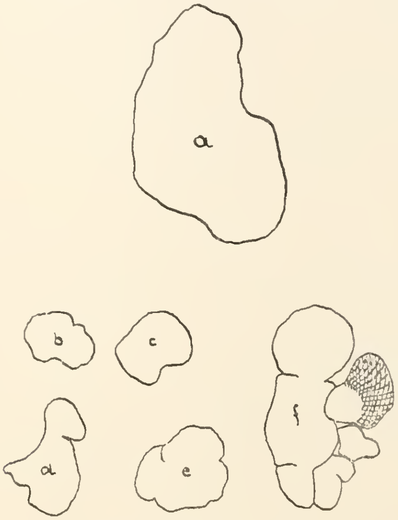

FIG. 6. Thyroid and follicles composing it, of specimen No. 47 in Table I., an animal which was examined one half hour after the first skin shedding. The whole thyroid gland was composed of only five follicles, one especially large and making the largest part of the entire organ.

Outlines drawn with the aid of a camera lucida, at a magnification of × 33 (Zeiss Binocular Dissecting Microscope, Oc. 2, Obj. a_2).

a, outlines of largest optical section of the whole thyroid gland.

b, *c*, *d*, *e* and *f*, outlines of the follicles composing the gland.

THE MODE OF THYROID GROWTH.

As may be calculated from the values recorded in column 8 of Table I. (area in cm^2.) the volume of the adult thyroid gland of *A. maculatum* is about 680 times as large as the volume of the thyroid of a larva at the time at which the first toes are developed.

This considerable growth might take place in two ways, either by an increase in the size of the follicles or by an increase in the number of the follicles.

The diverticulation of the follicles, increasing with the size of the gland, the presence of follicles which consist of several component follicles communicating often only by a narrow stalk and finally the presence of relatively very small follicles in the thyroids of practically every stage, if taken together, would suggest that the follicles develop buds which, after attaining a certain size, separate from the mother follicles. If this assumption were correct, the number of follicles should increase with the size of the gland and more follicles should be found in the adult than in the larval gland. In reality the number of follicles stays unchanged. *The growth of the thyroid is effected entirely by an increase in the size of the follicles.*

Nevertheless it could be imagined that the follicles composing a particular thyroid at a certain stage are not the same follicles composing the same thyroid at another stage. The follicles could divide continuously without increasing in number, if just as many follicles disintegrate as new ones are forming. Although under certain experimental conditions, as will be described in a subsequent article, disintegration of many follicles may be produced, positively no atrophic or degenerating follicles were ever found in the normal thyroid of *A. maculatum.* This observation is fully in accord with the condition found through histological study in the thyroid of *A. opacum.*

Although the shape of the follicles suggests strongly a process of budding and cleavage of larger into smaller follicles, it is obvious that in reality *separation of the bud-like diverticula from the mother follicles and cleavage of larger follicles into smaller follicles does not take place.*

The presence of bud-like diverticula and of composite follicles may be explained in two different ways. The diverticula may form as an outgrowth from the wall of the original follicles or they may be the result of fusion of smaller follicles with larger follicles. Histological study of the thyroid of *A. opacum* showed convincingly that follicular fusion plays a very important rôle in the growth of the thyroid of this animal. Conditions in *A. maculatum* are somewhat less favorable to analyzing this phase of the problem. Several facts, however, suggest, that in the spotted salamander processes of follicular fusion are going on during a large part of the animal's life. As pointed out, small spherical follicles are present in the thyroids of almost every stage. A complete analysis of the problem hinges around the question whether the primary follicles found in any particular stage of an individual thyroid gland are the same as those present at a later stage or whether new primary follicles are developing continuously during the growth of the thyroid. The primary follicles retain practically the same size from an early larval stage up to the winter of the second year of the animal's life. If the primary follicles found during the second winter are the same as those present at the early larval stages, it would mean that they have not been taking part in the general follicular growth. It would be difficult to explain this peculiarity. Neither pressure nor lack of nourishment can be invoked as the cause of it; the primary follicles are located almost invariably at the periphery of the organ and are frequently in close vicinity of large vessels. Moreover the primary follicles do not by any means lack the potential ability of growing; on the contrary they may finally attain a considerable size as may be seen in the thyroids of completely adult, sex-mature animals.

If the primary follicles seen in the thyroid during the later stages are not the same individuals as those seen at the earlier stages, then it would mean that new primary follicles are forming continuously during the growth of the thyroid, but do not increase in number, because a corresponding number fuse with the follicles already present, either while they are still small and spherical (bud-like diverticula) or after they have reached a larger size (composite follicles). Two facts were observed, which would fit in well with this interpretation. As has been mentioned above, follicles are found frequently, which consist of several large component follicles communicating with each other sometimes merely by a small hole. Since cleavage of follicles does positively not occur, this condition may indicate a fusion in its earliest beginning. Particularly suggestive are such follicles which are in closest apposition along a considerable surface, but communicate nevertheless only by a small opening in the adjacent walls (Fig. 1, *p*). The small spherical follicles present very frequently a relation to the large follicles, which is very similar to the relation of the bud-like diverticula and is different from it only by the lack of communication (Fig. 1, *f*).

The other circumstance of interest in this connection is the frequent presence of tiny solid cell masses in thyroids of nearly every stage. These cell masses are difficult to find in the macerated glands and it may be for that reason that they have been overlooked in many glands. With the method of maceration a minute analysis of these cell masses is impossible. It should be pointed out, however, that they are epithelial in nature, distinctly different from the connective tissue as well as from the clusters of red blood corpuscles. It is believed that they correspond to similar cell masses found in histological thyroid sections of *A. opacum* and called "reserve cell masses" in a previous paper (11). Microscopical study of these cell masses in the marble salamander showed that they are the source for continuous development of small young follicles. Most probably the same condition prevails in the spotted salamander; the reserve cell masses split off, during a large part of the animal's life, new primary follicles which in turn fuse with the old follicles. The increase in the size of the smallest follicles as observed in the glands of adult animals indicates that in these glands development of new primary follicles has ceased and no further fusion of the older primary follicles with the large follicles takes place.

These facts suggest that *at least a certain number of diverticula are the result of fusion of smaller follicles with larger ones.*

It was observed that in *A. opacum* intracellular colloid globules may enlarge within the cells of the follicular wall, come to lie free within the epithelial wall and finally fuse with the main mass of intrafollicular colloid (11). While they lie free in the epithelial wall they may, sometimes, form a small, bud-like protrusion on the outside of the follicle. Possibly some of the very smallest diverticula in *A. maculatum* are formed by such intracellular colloid droplets.

It is of interest to note that Heidenhain found separation of buds from the mother follicles and cleavage of large into small follicles entirely absent in the thyroid of mammals. Our findings in the thyroid of *A. maculatum* corroborate entirely Heidenhain's observation in the thyroid of mammals. As mentioned above, Heidenhain distinguishes two types of thyroids, an association and a dissociation type. The thyroid of *Ambystoma maculatum* belongs to the dissociation type. The separation of the follicles, however, is absolutely complete. The bud-like diverticula of the large follicles, if they occur at all, are, according to Heidenhain, primary follicles not yet isolated. In *A. maculatum* the bud-like diverticula cannot be interpreted this way; as indicated by the perfectly smooth and spherical shape of the primary follicles isolation takes place at an early stage.

Fusion of follicles isolated previously was not observed by Heidenhain in the mammalian thyroid gland; it takes place, according to this author, only between the lumina of follicles which have not been separated before. As described in a previous article (11) this kind of fusion is not uncommon in the marble salamander. In the spotted salamander, it seems, fusion is accomplished between follicles which have been really separated before. We are in doubt, however, that in the material and with the method used by Heidenhain a secondary fusion of previously separated follicles could be detected, if it did exist.

Epithelial reserve cell masses from which follicles are split off continuously were not observed by Heidenhain in the mammalian gland. He considers the small follicles of the cattle thyroid as the primary result of the general dissociation taking place at a very early stage. According to his views large numbers of the small primary follicles remain permanently unchanged, from the time of their separation till old age. In *A. punctatum* these primary follicles are absent in the adult glands indicating that finally the primary follicles begin to grow too.

Exceptional Cases of the Follicular Pattern.

In *A. opacum* the follicular pattern shows a very definite relation to metamorphosis. Two types of follicular design are characteristic. As the follicles begin fusing with each other shortly after the completion of the limb-development, the thyroid consists, some time before metamorphosis takes place, for the most part of a large, tube-like follicle distended greatly with colloid. The other characteristic pattern is found just after the first skin shedding. At this time the large, tube-like follicle collapses nearly in its entire extent, as the colloid is released from its lumen, and then forms a nearly solid and continuous mass of cells.

As will be evident from the facts described above neither of these conditions was found to be an integral part of the developmental cycle of the thyroid of *A. maculatum.* Nevertheless we found two thyroids which recall in a most conspicuous manner the structure characteristic for the thyroid of *A. opacum.*

The first case is represented by an animal of series CCLXII., animal No. 47 in Table I.; its thyroid was examined one half hour after the first skin shedding. Only one of the two thyroids was macerated. It consisted of only 5 follicles (Fig. 6). Small spherical follicles were missing entirely. Four of the five follicles were of good size, but did not show anything unusual. The fifth follicle, however, was excessively large, making up the largest portion of the entire gland. It consisted of seven component follicles of rather smooth outlines; one of these was partly solid. On the whole this follicle resembled the large baglike follicle found normally in the thyroid of *A. opacum* and developing there by fusion of the smaller follicles. In *A. maculatum* this condition was found only once among 120 animals.

The second case is represented by the thyroid of animal No. 54 in Table I., which was killed one day after the first skin shedding. This thyroid consisted almost in its entirety of a solid mass of cells, devoid of any lumen and colloid. In addition 11 smaller masses were isolated. But only 4 of them were follicles of vesicular nature; the other seven were solid cell masses; it was suspected that they had been broken off, in the process of dissecting the macerated gland, from the main mass. This thyroid resembles closely the condition of the thyroid found normally in *A. opacum* shortly after the first skin shedding and called in a previous paper (11) "stage of colloid release." In *A. maculatum* it was found only once among 120 animals. But in another species, *A. jeffersonianum*, of which only few thyroids were examined by the maceration method, exactly the same condition was met with in an animal which was examined one day after it had been placed on land (CCXXXIII. 7). Animals of the same series from which the *A. maculatum* described above had been taken were examined more than one year after the first skin shedding; none of them showed this condition of

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almost complete continuity of the epithelium and lack of colloid. It is therefore certain that it was not a peculiar thyroid structure characteristic for the offspring of one particular female.

It may be said, then, that although as a rule the follicular pattern of the thyroid of *A. maculatum* does not show any striking changes corresponding to certain functional changes, such changes may develop in rare cases. They resemble the changes taking place regularly in *A. opacum*. It should be noted especially that while the development of one particularly large tube-like follicle is not an integral phase in the functional cycle of the thyroid of the spotted salamander, it does occur occasionally.

On the whole the differences of the follicular pattern of the thyroid, existing between two so closely related species as *A. maculatum* and *A. opacum*, are very striking. Yet the occurrence of the two exceptional cases of follicular pattern in *A. maculatum* shows, that in the thyroid of this species the ability of developing the follicular pattern of *A. opacum* is potentially present.

Comparison of the thyroids of the two species of salamanders examined furnishes, we believe, further evidence to show that the plasticity of the endocrine system is very considerable in the group of amphibians.

Variability of the Number of Follicles.

Owing to the extreme variability of the number of follicles it is difficult to determine whether or not a change in the number of follicles can be effected by any particular experimental procedure. Nevertheless, as we have seen in experiments to be described in a subsequent paper (13), it is possible to change so considerably the number of follicles, that the degree of the change exceeds the degree of variability. In the present article we will discuss only such variations the causes of which are not entirely understood.

As was pointed out, when reference was made to Table I. the number of follicles varies between 5 and 72, if all of the animals examined are included. If, however, animals of the same parentage are compared, the variability is considerably less, as may be seen from an inspection of Table III. All animals

TABLE III.

Series CCLXXI.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm.^{2} Area.	Area, in cm.^{2} of Largest Optical Section of Thyroid at a Magnification of × 33.
3	2.5 toes; 1 balancer left.	7.5	10	—	—	0.16
1	2.5 toes; no balancer left.	8.0	10	—	—	0.16
7	3.0 toes.	8.4	12	—	—	0.12
9	3.5 toes; hind limb buds.	9.5	21	—	—	0.33
10	3.5 toes; hind limb buds.	9.6	21	—	—	0.25
11	4.0 toes; hind limb buds split in two.	9.6	28	—	—	0.41
12	6.0 toes.	11.1	29	—	—	0.14
14	8.5 toes.	15.1	44	*38*	42	0.9
16	Larval.	21.4	60	*34*	16	2.2
19	Larval.	21.5	63	*58*	25	2.3
20	Larval.	22.2	63	*24*	13	1.9
17	Larval.	22.8	62	*31*	13	2.5
18	Larval.	22.8	63	*38*	16	2.4
a 1	E.pr.sl.	25.7	75	*42*	17	2.55
a 2	E.pr.sl.	27.9	77	*51*	16	3.1
a 4	E.pr.d.;[1] yell.N.	31.1	77	*35*	15	3.4
a 5	Pieces 1 day; yell.N.; Op. very shall.	35.9	88	*35*	13	2.75
a 6	Yellow spots; Op. and G. closed.	35.6	89	*65*	12	5.4

TABLE III. (*continued*).

Series CCLXXI.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm.^{2} Area.	Area, in cm.^{2} of Largest Optical Section of Thyroid at a Magnification of × 33.
a 3	Just shedding Sk.; Op. and G. closed.	27.8	77	*30*	10	2.95
*a*12	Just Sh.Sk.; Op. and G. partly open.	—	91	*28*	9	3.05
*a*14	Sh.Sk. 0 days; Op. and G. nearly closed.	35.2	95	*24*	8	3.2
a 7	Sh.Sk. and L. 1 day; Op. and G. closed.	33.8	89	*50*	12	4.2
a 9	Sh.Sk. and L. 2 days; Op. and G. closed.	31.6	89	*52*	11	4.6
a 8	Sh.Sk. and L. 2 days; Op. and G. closed.	33.5	89	*42*	8	5.2
*a*13	Sh.Sk. and L. 2 days; Op. and G. closed.	34.8	92	*44*	6	7.9
*a*10	Sh.Sk. and L. 3 days; Op. and G. closed.	32.2	89	*57*	13	4.25
*a*11	Sh.Sk. and L. 4 days; Gills absorbed.	29.6	89	*43*	10	4.45
*a*15	Sh.Sk. and L. 6 days.	33.1	96	*42*	7	5.7
*a*18	Sh.Sk. and L. 11 days.	32.2	100	*63*	15	4.2
*a*19	Sh.Sk. and L. 11 days; Eats 1 day.	33.1	101	*36*	15	2.45
*a*20	Sh.Sk. and L. 12 days; Eats 4 days.	34.9	101	*50*	9	5.4
*a*16	Sh.Sk. and L. 13 days.	31.1	96	*36*	12	3.05
*a*17	Sh.Sk. and L. 13 days.	31.6	96	*44*	10	4.45
*a*21	Sh.Sk. and L. 13 days.	35.2	101	*26*	6	4.5
*a*22	Sh.Sk. and L. 17 days.	35.3	102	*47*	14	3.6
*a*23	Sh.Sk. and L. 20 days.	31.8	104	*72*	25	2.9
*a*24	Sh.Sk. and L. 189 days; killed February.	40.5	279	*39*	10	4.1
*a*25	Sh.Sk. and L. 197 days; killed April.	46.0	328	*36*	10	3.5
Average				*42.3*	13.5	

All animals included in this table are the offspring of the same female.
For explanation and abbreviations see explanation to Table I.

included in this table are the offspring of the same female. The average number of follicles in this brood is 42, the smallest number was 24, the largest 72. Among 31 animals there were seven possessing more than 50 follicles, and only three whose thyroids contained more than 60 follicles.

In Table IV. are compared two series which were raised under the same conditions, but were the offspring of two different females. There is a marked difference in the follicular number between the two series. The average numbers show a difference of 15.

In Table V. "lot *a*" of the series CCLXXI. is compared with "lot *b*" of the same series. Of the thyroids of the 8 animals of "lot *b*" seven (87.5 per cent.) contain more than 40 follicles, three (37.5 per cent.) more than 65 follicles; in "lot *a*" the thyroids of only two animals (8.3 per cent.) among 24, contain more than 65 follicles, 15 (62.5 per cent.) more than 40 follicles. The average number of follicles in "lot *a*" is 44, in "lot *b*" 54. Both lots are the offspring of the same female. But in "lot *a*" the larvæ were isolated, at an early stage, into individual finger-bowls; individual attention was given to each animal both in regard to food and water. The larvæ of "lot *b*" were left together in one large dish; the supply of oxygen was poor and the food scarce. Only at the end of the larval period they were placed under conditions similar to those of "lot *a*." The follicular number of the animals raised under favorable conditions is lower than the follicular number of those raised under poor conditions.

In Table VI. "lot *b*" of the preceding series is compared with series CCLXII. As mentioned before, "lot *b*" consisted of animals raised under unfavorable conditions of food and oxygen. Series CCLXII. was composed of larvæ of unknown parentage, collected in one of the ponds of the vicinity of Woods Hole and brought to the laboratory only shortly before metamorphosis These animals were extremely well nourished, of large size and had lived under optimum conditions. The difference in the follicular number between this series and "lot *b*" of the series CCLXXI. is still greater than the difference between the well nourished "lot *a*" of series CLXXI. and the "lot *b*." In series

TABLE IV.

THE PROGENY OF TWO DIFFERENT FEMALES OF *Ambystoma maculatum*, RAISED UNDER THE SAME CONDITIONS, ARE COMPARED REGARDING THE NUMBER OF FOLLICLES OF THE THYROID GLAND.

Series CCLXXIV.						Series CCLXXI.*b*.					
Number.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Number of Follicles per cm². Area.	*Number of Follicles.*	Age in Days.	Body Length in mm.	Stage of Animal.	Number.
1	Larval.	20.0	65	*52*	43						
2	Larval.	21.3	66	*28*	22						
*a*1	E.pr.d.	29.2	92	*49*	14						
*a*2	E.pr.d.	32.3	92	*29*	7	14	*41*	79	27.2	E.pr.sl.; yell.N.	2
						17	*56*	87	30.6	E.pr.d.; yell.N.; Op.shall.	3
*a*3	Pieces 1 day; E.pr.d.; yell.N.	33.7	96	*32*	11	11	*72*	88	31.1	E.pr.d.; Op.shall.	5
						14	*47*	91	32.4	Just shedding; Op. and G. open.	6
						17	*43*	77	27.1	Just Sh.Sk.; Op. and G. closed.	1
3	Sh.Sk. 0 days.	22.5	68	*48*	19						
*a*5	Sh.Sk. and L. 1 day.	—	106	*26*	10						
						16	*71*	92	31.7	Sh.Sk. and L. 2 days.	7
						21	*67*	95	28.6	Sh.Sk. and L. 3 days.	8
						17	*32*	87	28.8	Sh.Sk. and L. 4 days.	4
*a*4	Sh.Sk. and L. 5 days.	35.4	100	*47*	16						
	Average			*39*	18	16	*54*				

TABLE V.

TWO SERIES OF *Ambystoma maculatum*, COMPOSED OF THE OFFSPRING OF THE SAME FEMALE, BUT RAISED UNDER DIFFERENT CONDITIONS, ARE COMPARED WITH EACH OTHER REGARDING THE NUMBER OF FOLLICLES OF THE THYROID GLAND.

Series CCLXXI.*a*.						Series CCLXXI.*b*.					
Number.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm.² Area.	Number of Follicles per cm.² Area.	*Number of Follicles.*	Age in Days.	Body Length in mm.	Stage of Animal.	Number.
1	E.pr.sl.	25.7	75	42	17						
2	E.pr.sl.	27.9	77	51	16						
4	E.pr.d.	31.1	77	35	15	14	41	79	27.2	E.pr.sl.; yell.N.	2
						17	56	87	30.6	E.pr.d.; yell.N.	3
5	E.pr.c.; yell.N.	35.9	88	35	13	11	72	88	31.1	E.pr.d.; yell.N.	5
6	E.pr.c.; Op. and G. closed.	35.6	89	65	12						
3	Just shedding; Op. and G. closed.	27.8	77	30	10	14	47	91	32.4	Just shedding; Op. and G. open.	6
12	Just Sh.Sk.; Op. and G. open.	—	91	28	9	17	43	77	27.1	Just Sh.Sk.; Op. and G. closed.	1
14	Sh.Sk. 0 days; Op. and G. open.	35.2	95	24	8						
7	Sh.Sk. 1 day; Op. and G. closed.	33.8	89	50	12						
9	Sh.Sk. and L. 2 days; Op. and G. closed.	31.6	89	52	11						
8	Sh.Sk. and L. 2 days; Op. and G. closed.	33.5	89	42	8	16	71	92	31.7	Sh.Sk. and L. 2 days; Op. and G. closed.	7
13	Sh.Sk. and L. 2 days; Op. and G. closed.	34.8	92	44	6						
10	Sh.Sk. and L. 3 days; Op. and G. closed.	32.2	89	57	13	21	67	95	28.6	Sh.Sk. and L. 3 days; Op. and G. closed.	8
11	Sh.Sk. and L. 4 days.	29.6	89	43	10	17	32	87	28.8	Sh.Sk. and L. 4 days.	4
15	Sh.Sk. and L. 6 days.	33.1	96	42	7						
18	Sh.Sk. and L. 11 days.	32.2	100	63	15						
19	Sh.Sk. and L. 11 days.	33.1	101	36	15						
20	Sh.Sk. and L. 12 days.	34.9	101	50	9						
16	Sh.Sk. and L. 13 days.	31.1	96	36	12						
17	Sh.Sk. and L. 13 days.	31.6	96	44	10						
21	Sh.Sk. and L. 13 days.	35.2	101	26	6						
22	Sh.Sk. and L. 17 days.	35.3	102	47	14						
23	Sh.Sk. and L. 20 days.	31.8	104	72	25						
	Average			44	12	16	54				

TABLE VI.

TWO SERIES OF *Ambystoma maculatum*, ONE RAISED UNDER OPTIMUM CONDITIONS (CCLXII.), THE OTHER ONE UNDER POOR CONDITIONS (CCLXXI.*b*), ARE COMPARED WITH EACH OTHER REGARDING THE NUMBER OF FOLLICLES OF THE THYROID GLAND.

Series CCLXII.						Series CCLXXI.*b*.					
Number.	Stage of Animal.	Body Length in mm.	Age in Days.	*Number of Follicles.*	Number of Follicles per cm². Area.	Number of Follicles per cm². Area.	*Number af Follicles.*	Age in Days.	Body Length in mm.	Stage of Animal.	Number.
6	Larval; yell.N.	36.0	125	*10*	3	14	*41*	79	27.2	Larval; E.pr.sl.; yell.N.	2
4	Late larva; E.pr.	37.3	125	*35*	7	17	*56*	87	30.6	E.pr.d.; yell.N.; Op.shall.	3
10	Late larva; E.pr.	37.4	127	*30*	7	11	*72*	88	31.1	E.pr.d.; yell.N.; Op.shall.	5
8	Begins Shedd.Sk.; Op.shall.	37.3	126	*37*	8						
7	Begins Shedd.Sk.; Op.shall.	42.0	126	*25*	3						
						14	*47*	91	32.4	Just shedding; Op. and G. open.	6
2	Just Sh.Sk.; Op. clos.; G. open.	42.4	126	*31*	4	17	*43*	77	27.1	Just Sh.Sk.; Op. and G. closed.	1
5	Sh.Sk. ½ hour; Op. and G. open.	40.1	125	*5*	1						
9	Sh.Sk. 3 hours; Op. very shall.	37.0	126	*44*	8						
11	Sh.Sk. and L. 1 day; Op. and G. clos.	36.1	128	*30*	7						
12	Sh.Sk. and L. 1 day; Op. and G. clos.	32.4	128	*37*	10						
1	Sh.Sk. 2 days; L. 1 day; Op. and G. clos.	40.9	120	*39*	5	16	*71*	92	31.7	Sh.Sk. and L. 2 days; Op. and G. clcs.	7
						21	*67*	95	28.6	Sh.Sk. and L. 3 days; Op. and G. clos.	8
14	Sh.Sk. 4 days; L. 3 days; Op. and G. clos.	41.4	130	*36*	4	17	*32*	87	28.8	Sh.Sk. and L. 4 days; Op. and G. clos.	4
	Average			*30*	6	16	*54*				

CCLXII. the average follicular number is only 30, while in "lot *b*" it is 54; there is a difference of 24 between the average follicular numbers of these two series. And it is again the series reared under better conditions, in which the follicular number is lower.

As far as we can trust these scanty figures it seems that the follicular number of the thyroid gland is influenced by hereditary and by environmental factors. Among the descendants of one particular female there may be more animals with conspicuously low or high numbers of follicles than among the descendants of other females. Animals growing up in a favorable environment have a lower number of follicles than those growing up in a less favorable environment.

We do not know what rôle the number of follicles might play in the function of the thyroid gland. Nevertheless it is known that certain pathological conditions are characterized by high or low numbers of follicles. It is of interest in this connection that the inclination towards a higher or lower number of follicles may be inherited.

SUMMARY.

1. In the species *Ambystoma maculatum*, as contrasted to the species *A. opacum*, the number of follicles does not show, as a rule, the characteristic changes related to metamorphosis and consisting in the formation, by fusion of follicles, of a large, tube-like or bag-like follicle filled with colloid. Nevertheless a large, bag-like follicle making the largest part of the entire organ was found in one animal among 120.
2. In the species *A. maculatum* the average number of follicles is 39.7.
3. The number of follicles in each thyroid remains constant during the entire life of the individual.
4. The growth of the thyroid is effected entirely by an increase in the size of the follicles.
5. The primary follicles are simple spherical vesicles.
6. As the follicles grow in size the surface becomes markedly irregular. The large follicles possess diverticula of varying number, size and shape or may be composed of two or more equally large and diverticulated component follicles.

7. Separation of the bud-like diverticula from the mother follicles or cleavage of the larger follicles into smaller follicles does not occur.

8. The bud-like diverticula and composite follicles are the result of fusion between adjacent follicles.

9. The source for the continuous formation of new follicles are probably small masses of epithelial cells found in many thyroids.

10. Owing to the fusion of the primary follicles, which goes on at the same rate as the formation of new follicles, the number of follicles remains constant.

11. In adult, sex-mature animals primary follicles cease to develop; those present do not fuse with larger follicles, but themselves begin to enlarge in size.

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REACTIONS OF AN ELASMOBRANCH (*SQUALUS SUCKLII*) TO VARIATIONS IN THE SALINITY OF THE SURROUNDING MEDIUM.

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A study of the changes which occur in fish when placed in a medium other than their natural habitat has attracted the attention of many investigators. Variations have been made both in the concentration and in the kinds of salts used and experiments of this nature have been conducted with teleosts and elasmobranchs. Our knowledge of the subject is, however, by no means complete, and a further investigation of certain phases was thought to be of value.

It is generally appreciated that elasmobranchs are usually less resistant to changes in salinity than are the teleosts. The former, therefore, make excellent subjects for an investigation of certain phases of the problem. *Squalus sucklii* was selected for the following investigation. This is a species of elasmobranch which has heretofore received little attention from physiologists.

In addition to observing the length of time this fish was able to live in certain solutions differing in amount and kind of salinity from its natural habitat an investigation was made of the changes in the gill movements, the heart beat, the amount of hemoglobin and the weight of the animal when taken from sea water and from the experimental solutions. A comparison was also made of the weight of the liver, spleen and pancreas taken from fish that had died in the various media.

The fish used in this research were taken on a set line from the Strait of Georgia near Departure Bay, Vancouver Island, and usually from a depth of 30 yards. The fish were handled very carefully but some injury always resulted from this method of capture. However, good specimens usually would live for a week when kept in cages fastened to a float in sea water despite the fact that they were not fed during this time and that the salinity of this water was lower than that of their natural habitat. The fish were taken during the months of June, July and August of 1926 and a total of 219 were obtained suitable for this investigation. The fish were kept in a cage fastened to a float in sea water and were usually used within a few hours after being taken from the set line, and always within 15 hours of the time of capture.

For the purpose of comparison some physical characteristics are given of the solutions with which this investigation is concerned (Table I.).

METHODS.

In this investigation, the following media were used: (*a*) distilled water, (*b*) tap water, (*c*) tap water which had been given a pH of 8.4 by the addition of NaOH solution, (*d*) NaCl, in distilled water, (*e*) NaCl, $CaCl_2$ in distilled water, (*f*) NaCl, $CaCl_2$, KCl in distilled water, (*g*) NaCl, $CaCl_2$, KCl, $MgSO_4$ in distilled water, (*h*) sea water to which NaCl, $CaCl_2$, KCl, $MgSO_4$ had been added. In each case the quantity of the salt added was the amount required to give a concentration of the cation approximately the same as that found in sea water. When distilled water was used it was freshly made, cooled and then shaken with air. The water was obtained from a copper still but this is considered to be of negligible importance. The various solutions were frequently agitated during the course of the experiment and the fish were changed to a fresh medium of the same kind if the experiment continued for more than an hour.

Since it was desired to have conditions approximate as nearly as possible those which a fish would encounter if it were to swim naturally into the solution used, the fish were not "wiped." Before being placed in the solution they were allowed to hang head downward for one half minute, and then weighed. A slight amount of bleeding was produced by introducing the point of a scalpel having a thin blade into the caudal artery at the base of the caudal fin. Samples of blood were taken for the determination of the amount of hemoglobin and then the fish were placed in the desired solution.

TABLE I.

Medium.	pH.	Temperature Degrees C.	Density.	Total Solids gm. per Liter.	Oxygen Content cc. per Liter.	Grams per Liter.			
						NaCl	$CaCl_2$	KCl.	$MgSO_4$.
Water from which fish were captured	8.4	10.3[1]	1.0218[1]		4.41[1]	27.37[1]			
Water in cage where fish were kept	8.4	16–18	1.0184[1]	28.72	5.43[1]	22.17[1]			
Distilled water	7.0	16–18	1.0000	0		0	0	0	0
Tap water	6.9	16–18	1.0003[1]	0.087	5.32	Trace			
Tap water pH 8.4	8.4	16–18							
NaCl solution	7.0	16–18		27.0		27	0	0	0
NaCl, $CaCl_2$ solution	7.0	16–18		28.164		27	1.164	0	0
NaCl, $CaCl_2$, KCl solution	7.0	16–18		28.914		27	1.164	0.75	0
NaCl, $CaCl_2$, KCl, $MgSO_4$ solution	7.0	16–18		35.414		27	1.164	0.75	6.5
						Added to sea water			
Sea water with added salts	7.0	16–18		64.134		27	1.164	0.75	6.5

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[1] C. C. Lucas, personal communication.

It is well recognized that the effectiveness of a stimulus is partly dependent on the rate at which it is applied, therefore the sudden immersion of the fish in the solution should result in a maximum effect. Also, the injuries which the fish received incident to capture might alter slightly the results of an experiment.

After a variable length of time, the respiratory movements became slower and weaker. As the paralysis of the respiratory movements became more marked some struggling usually occurred and finally respiration completely failed. When respiration ceased, as was evidenced by cessation of movements of the spiracle, the time was noted, the fish was removed from the water, suspended head downward for one half minute as before, and reweighed. Blood was again taken for the determination of hemoglobin. The fish was placed on a tray, the heart exposed and if found beating, as was practically always the case, the time when the cardiac movements ceased was noted. The liver, spleen and pancreas were then exposed, freed from adjacent tissue and weighed.

Observations were made of the respiratory movements of all the fish used while in the various solutions but a series of thirteen fish were used in making a more complete study of the effect of tap water on the nature of the respiratory and cardiac movements. Freshly caught fish were weighed and then fastened to a wooden frame. The frame was submerged in a tank of sea water in such a manner that all of the fish, with the exception of the ventral surface directly over the heart, was covered by the water. A median incision was made in the exposed area, the tissue at the sides of the incision was retracted in such a manner that the heart was exposed, but the entrance of water to the wound was prevented. By means of a thread, the apex of the ventricle was attached to a heart lever arranged to write on a kymograph drum. A second heart lever was attached to one of the gill slits and arranged to record respiratory movements on the same drum. A record was made of the respiratory and cardiac movements of the fish in sea water. This water was then quickly drawn out of the tank and replaced by tap water and a second record was made. After the fish had been in tap water for thirty minutes, a third record was made and then the water was replaced by fresh tap water. A fourth record was made after the fish had been in the tap water for fifty-five minutes. After the fish had been in the tap water for sixty minutes, it was removed from the water and killed by destruction of the central nervous system.

In determining the normal weight of the liver, spleen and pancreas 103 fish (34.9 per cent. females) of various sizes were used. These were fish that died in sea water either shortly before lifting the set line or soon after being placed in the cages. The fish were held head downward for one half minute to drain and then weighed. The abdominal cavity was opened by a median incision. The liver, spleen and pancreas were each dissected from the adjacent tissue and immediately weighed. From the figures so obtained, the weight of each organ per gram of fish was calculated.

Results and Discussion.

Toxicity of Media.—As a result of the studies of many investigators [for a review of this subject see Garrey (1, 2), Scott (3, 4), Macallum (5)] it has been shown that the osmotic pressure of the blood of a fish is rarely the same as that of its natural habitat. This is made possible by the relative impermeability of the integument, alimentary tract mucosa and of the gill membranes. The kidneys also play an important rôle in keeping the blood composition constant. However, when the fish is transferred to a medium having a different osmotic pressure the impermeability of the gill membranes is reduced and a passage of water and salts occurs which can not be entirely counter-balanced by the activity of the kidneys. This leads to a partial equalization of the osmotic pressures of the solutions separated by the gill membranes. In general, this equalization occurs more rapidly and more completely in elasmobranchs than in teleosts. Toxicity of a solution appears to some extent to be related to the degree to which it differs in osmotic pressure from that of the natural habitat of the fish. Therefore, teleosts will frequently survive changes in osmotic pressure of the external medium which would be fatal to elasmobranchs.

Distilled water has usually been found to be very toxic for fish, Ringer (6), Wells (7), but Garrey (2) found that fish can live in this medium for weeks and he quotes a similar observation made by Loeb. Garrey (2) has likewise corroborated the findings of Ringer (6) (8) that the toxicity of distilled water is reduced by the addition of a small amount of salts; for example, fresh water is less toxic than distilled water.

Many physiologists Loeb (9), Ringer (6), Garrey (2) have demonstrated with a number of species of marine and fresh water teleosts the mutual antagonism and progressive decrease in toxicity as Ca, then K, and finally Mg ions have been added in definite proportions to NaCl solutions. The usual explanation offered is that these ions exert their protective action by reducing the injurious effect which the individual ions have on the permeability of the gill membranes to salts and water.

In so far as I have been able to ascertain no previous investigation has been made on the effects of physiologically unbalanced solutions, with the possible exception of fresh water, on any elasmobranch or the effect of physiologically balanced solutions on *Squalus sucklii*.

In determining the effect of unbalanced, partially balanced and completely balanced solutions on dogfish it was found, that although the differences in the toxicity of the solutions used were not very great (Table II.), the results in general agreed rather well with those obtained by the other investigators. Using the time required for failure of the respiration as a guide to the toxicity, the solutions in order of their decreasing toxicity are Na > distilled water > Na, Ca, K, Mg, > Na, Ca, > Na, Ca, K. Since the solution containing the Na, Ca, K, and Mg ions was in substance an artificial sea water, it is difficult to explain its apparently high toxicity.

Using failure of the heart as a guide to the toxicity, the results are distilled water > Na > Na, Ca > Na, Ca, K, Mg > Na, Ca, K. The solution made by adding NaCl, $CaCl_2$, KCl, and $MgSO_4$ to sea water so that the cations would be present in approximately twice their normal concentration was a balanced solution. Therefore it is interesting to note that this solution as regards its effect on respiratory or cardiac movements was the

TABLE II.

Solution Used.	Number of Fish Used.[1]	Ave. Duration of Movements (in Minutes).		Ave. Change in Hemoglobin Gm. per 100 cc. Blood.	Ave. Percentage Change in Weight.
		Respiratory.	Cardiac.		
Distilled water	24	73	100	−0.613	+1.75
Tap water	23	113.2	144.7	−0.323	+3.66
Tap water pH 8.4	4	87.5	128	−0.487	+6.7
NaCl solution	10	71	137.7	−0.046	−1.09
NaCl, $CaCl_2$ solution	5	89	138	−0.035	−0.132
NaCl, $CaCl_2$, KCl solution	7	125	165	−0.0057	−2.379
NaCl, $CaCl_2$, KCl, $MgSO_4$ solution	6	88.3	153.3	+0.108	−0.032
Sea water with added salts	6	53.3	83.3	+0.345	+3.46

[1] Does not apply to determination of hemoglobin.

+ Indicates increase, − indicates decrease.

most toxic solution used. Experiments of Loeb and Wasteneys (10), Garrey (2) and Portier and Duval (11) also indicate that a balanced solution may be toxic when the concentration exceeds a certain limit.

The water referred to as tap water was a ground water caught in a small private reservoir and supplied to the Pacific Biological Station. It may be considered as similar to that which fish would encounter if they were to swim to a point above tide water in the streams around Departure Bay. This water had a very low salinity but it is quite possible that the cations Na, Ca, K and Mg were all present.

Tap water was found to require a longer time to produce cessation of respiration or cardiac failure than any of the experimental solutions with the exception of the Na, Ca, K solution, an observation which would strengthen the conclusion that toxicity is not a simple question of osmotic pressure. Since the dogfish continued to breath for an average of 113 minutes in tap water and remained active during most of this time, they probably could escape from a fresh water stream even if they were to swim into it above tide water level. As in the case of the salmon, Greene (12), the resistance of the dogfish to fresh water might be increased if it were to ascend the stream very gradually.

Scott (3), however, has shown that dogfish may be permanently injured by solutions whose osmotic pressure is markedly different from that of the normal habitat. This investigator has shown that when specimens of *Mustelus* were placed in either a hypotonic or hypertonic solution, the freezing point of the blood returned to normal either slowly or incompletely after being returned to sea water depending on the length of time the fish remained in the abnormal solution and the degree to which this solution differed from normal sea water. A similar conclusion may be drawn from the results obtained in this investigation for it was observed that after being left in distilled water or tap water until a disturbance of the respiration was observed, the condition of *Squalus sucklii* did not improve when transferred to sea water. The specimens of *Mustelus* used by Scott (3) in nearly every case died in less than 100 minutes when immersed in fresh water. Although it is likely that the tap water used in the experiments here described contained less salts than the fresh water used by Scott, *Squalus sucklii* (Table II.) continued to breath for an average of 113.2 minutes and cardiac failure occurred after 144.7 minutes.

Chidester (13) quotes the work of many investigators, from which it is to be concluded that fish are very sensitive to changes in the pH of the medium and, moreover, that the toxicity of a solution is less, the nearer it approaches the pH of the natural habitat. It was therefore considered of interest to determine whether the toxicity of tap water (pH 6.9) would be decreased by making the concentration of the hydrogen ion correspond with that of the normal sea water. For this purpose, NaOH was used rather than Na_2CO_3 so that the variation in the salinity would be slight. Contrary to expectations it was found (Table II.) that the water at pH 8.4 was more toxic than at 6.9. As will be shown later, the toxicity of abnormal media appears to be related to a depression of the respiratory center. It is therefore possible that the fish lived longer in the tap water pH 6.9 because this solution, being of an acid nature, was less depressant to the respiratory center than the tap water pH 8.4. It is also possible that the NaOH exerted a toxic action in some manner other than through an alteration of the pH. As will be shown later, the fish in this solution underwent a marked gain in weight, a fact which would indicate that the solution was very injurious to the gill membranes.

It was impossible to demonstrate any relation between the size of dogfish and their resistance to changes in salinity. Similar reports have been made for teleosts by Young (14) but Bert (15) maintained that the larger fish are more resistant. The fact that most of the large females used by the author were pregnant may be used in explaining why these fish were not more resistant.

Bert (16) has reported that when fresh water fish were transferred to sea water, circulatory changes occurred in the blood vessels of the gills so that the appearance of these structures was decidedly changed. In my comparison of the gills of dogfish that had died in sea water with those of fish that had died in the experimental solutions no constant variation in the appearance was observed.

When the dogfish were placed in the experimental solutions their behavior was in all cases, much the same. They usually remained quiet, giving no indication that the medium was obnoxious to them, but they moved energetically when disturbed. A white slimy material collected in the water and on the bodies of the fish after they had been in the solution for some time, and in some cases opisthotonos, most marked in the region of the neck, was observed. Shortly before and after the respiratory movements ceased, struggling movements frequently occurred. The eyes were usually opaque by the time respiration ceased. The significance of the previously mentioned slime is not known, but it may indicate that the solutions had an action on the integument of the fish, perhaps stimulating secretion from the gland cells of the integument. The formation of slime in teleosts associated with changes in the environment has been reported by Young (14) and by Bert (16).

Many pregnant fish were used in the experiments but in no case did abortion occur as the result of introducing the fish into the experimental solutions. On opening the abdominal cavity of these fish, after cessation of the heart beat, the embryos were in all cases found to be dead. Several pregnant fish taken directly from sea water were killed by destruction of the central nervous system. They were allowed to remain undisturbed for an hour or more and then the embryos were examined. In most cases they were found to be alive and active. The cause of death in embryos following the introduction of the mother into an abnormal medium is not known. Asphyxia and dilution of the blood probably play minor rôles in this phenomenon.

Gill Movements.—A gradual decrease in rate and amplitude of the respiratory movements followed the introduction of *Squalus sucklii* into a medium of abnormal salinity. Until the fish had been in the medium for about half the time they survived the change this decrease was usually slight and in some cases was absent, but during the latter half of the experiment the decrease in both rate and amplitude was more rapid.

For thirteen fish in sea water, following exposure of the heart, the respiratory rate varied between 18 and 73 but averaged 41.6 to the minute. The fish gave little or no evidence of injury as the result of the operation and the results obtained are believed to approximate rather closely those to be observed in the normal intact animal. One hour after placing the fish in tap water, the respiration of five had ceased and the respiratory rate of the remainder averaged 31.7 per minute.

Lyon (17) reports that the normal respiratory rate of the shark under experimental conditions varies between 18 and 30 per minute with an average rate of 23. A specimen of *Mustelus* examined by Scott (3) had a respiratory rate of 59 per minute just as the change from sea water to fresh water was being made. After sixty-seven minutes in fresh water this fish made only 8 very feeble respiratory movements per minute. A specimen of *Squalus acanthias* observed by the same investigator breathed while in sea water at the rate of 14 times per minute. Greene (12) states that the respiratory rate of the salmon varies between 60 and 120 per minute. Parker (18) records 35 to 40 respiratory movements in the normal specimen of *Mustelus* resting in sea water and 50 to 55 per minute in the fish swimming slowly.

Scott (3) noted that the respiratory and the heart rate in *Mustelus* were at times equal but they appeared to be little correlated. Lyon (17) found evidence to indicate that the heart of the sand shark normally takes its rate from the respiration. In the present investigation it was observed that in *Squalus sucklii* the respiratory rate and cardiac rate seemed to be unrelated (except as considered later, when struggling or gill cleaning movements occurred). They were rarely equal and usually the respiratory rate was between two and three times as rapid as the heart rate, except shortly before the death of the animal when the respiratory rate was markedly decreased while the heart rate had changed but little.

Hyde (19, 20) has noted slightly convulsive movements of the gills occurring in the normal skate and suggests that these may be for the purpose of forcing more water through the gill apertures and thus removing foreign matter. Lyon (17) observed that such movements occur in the shark when any foreign body or solution enters the mouth or when a manipulation of the body occurs. Scott (3) found that such movements became very marked in *Mustelus* some time after being transferred to fresh water but decreased in intensity and frequency before the death of the fish. He intimates that the injurious action of the fresh water on the gill membranes may be the cause of the increase in intensity and frequency of these movements. The gill cleaning movements did not occur according to Scott (3) when similar experiments were carried out on *Squalus acanthias*.

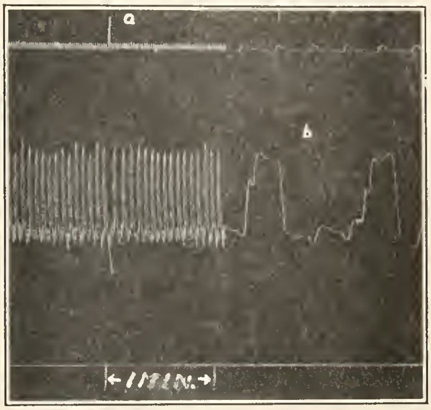
Movements similar to those described were noted in *Squalus sucklii*. These appear to be a normal movement and were observed while the fish were in sea water or in one of the experimental solutions. A more detailed study was made of these movements as they occurred when the fish were in tap water. With *Squalus sucklii* in tap water the gill cleaning movement may begin during any phase of the respiratory movement and consists of a single or more rarely two or three vigorous movements of the gills; the normal rhythm and amplitude is then regained. There is no preliminary movement preceding the gill cleaning movement. In some fish the gill cleaning movements may not be observed for some time but usually they occur at fairly regular intervals of one or two minutes. With certain fish, the intervals between these movements decreased to reach a minimum about one half hour after immersion in tap water and then decreased in frequency until respiration ceased. This was not of constant occurrence, for cases were observed where the movements disappeared after the immersion of the fish in tap water. The amplitude of the gill cleaning movements was not increased by immersion of the fish in fresh water. In fact it could not be definitely stated that immersion of the fish in fresh water produced any constant variation in these movements.

During the ventricular diastole following the gill cleaning movement, or more rarely during the second ventricular diastole, the heart apparently loses tone and marked dilatation occurs, the heart loses a beat and then quickly resumes the normal tone and rate. Scott (3) observed a similar change in *Mustelus* and suggested that "the cardiac spasm" is an instance of reflex inhibition of the heart beat due to the cardiac inhibitory center being stimulated by impulses from the sensory nerves. Reflex cardio-inhibition does readily occur in fish, Lyon (17), Greene (12), but because of its rhythmic nature and for other reasons the explanation of Scott does not appear to apply to the movements which I observed in *Squalus sucklii.* I am, however, in accord with his conclusion that the respiratory convulsions do not produce the peculiar cardiac movements, but that the two processes have the same cause.

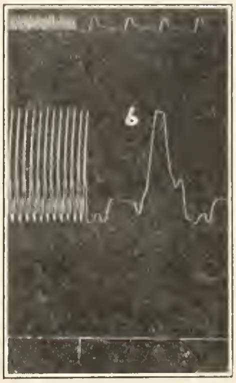
Distinction should be made between the movements described above and a somewhat similar movement observed in *Squalus sucklii.* This consisted of a spasm or series of struggling movements of the skeletal muscles but also involved the heart and gills. They appeared to occur during any part of the interval during which the fish were in the solutions and to originate in any external stimulus; occurring usually when the fish was touched or the water agitated. Several fish in a tank would remain quiet for a long interval, then a sudden movement of one individual would usually result in struggling movements among the others. These movements were more easily initiated after the fish had been in the solution for some time and especially just before and after respiration had ceased, that is, when the oxygen want might be expected to be greatest. The effect of these movements on the cardiac and respiratory movements was more pronounced but otherwise similar to those described as gill cleaning movements.

Although these movements would be likely to have a gill cleaning effect, this probably was not their primary object. They appeared to be the response given by the fish to a sensory stimulus after an increase in excitability had occurred. This increase in excitability may have been the result of the accumulation of CO_2 in the tissues due to the asphyxia which followed

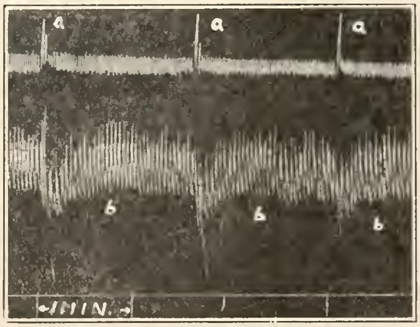
Simultaneous Records of Respiration (upper line) and Heart Beat (middle line).



Fish in sea water, (*a*) gill cleaning movement without a tonus wave in the record of the heart beat, (*b*) record on rapid drum.



After fish had been in tap water for 54 minutes, (*b*) record on rapid drum.



Record from fish after 6 minutes in tap water, (*a*) gill cleaning movements with (*b*) tonus waves.

the introduction of the fish into an abnormal medium. Bert (16) has described the agitation and irregular respiration occurring in fresh water fish transferred to sea water and Young (14) has observed that when fresh water teleosts were placed in a solution whose salinity differed from that of their natural habitat the fish were restless and easily excited before death. Wells (21) has noted a somewhat similar condition in fish dying in a solution containing a high concentration of carbon dioxide and a low concentration of oxygen. This apparently is the same condition as that described above for the dogfish. No other forms of irregularities in the respiration of the dogfish were noted. Cheyne-Stokes rhythm is at times observed in cases of anoxemia, I therefore expected that it might occur while the fish were in the experimental solutions but it was not observed. Lyon (17) has noted this type of respiration occurring in the shark shortly before death.

Cause of Death of Fish in Experimental Solutions.—Perhaps the most noticeable change which occurred in the dogfish when placed in the experimental solutions was a gradual but progressive slowing of the respiration terminating finally in a complete cessation of the respiratory movements. Since this change occurred with all of the solutions used, it does not appear to be the result of the presence or absence of any of the salts used nor of the pH of the solution. The rate at which failure of respiration becomes complete as shown in Table II. does vary with the salt or combination of salts used. This has previously been considered.

Many investigators studying the effect of variations in salinity have noted the injurious effect on the respiration and Lyon (17) has noted that the respiration ceases before the heart beat when sharks die in sea water, while Greene (12) from a study of the salmon concludes that the spawning act produces death through some more vulnerable channel than the heart and blood vessels. It may be a rule of more or less general application for fish that the respiratory apparatus succumbs more readily than the circulatory.

Assuming that the fish used in our experiments died from asphyxia, it is interesting to note that the solutions used contained

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about as much oxygen at the beginning of the experiment as did the sea water from which the fish were captured. The solutions were agitated frequently and it is not likely that any marked decrease in the oxygen content occurred during the experiment. Moreover Powers (22) has noted that the oxygen content of the normal medium in which fresh water or marine fish live can be decreased to a low level (between 1.7–0.4 cc. per liter) before the fish will exhibit oxygen want.

That anoxemia was an important factor in the death of the fish placed in experimental solutions has been the conclusion of many investigators. Indeed, it has been shown by Backman (23) that if the sea water in which dogfish live, with $\Delta = -1.88^\circ$ is diluted to $\Delta = -0.5^\circ$, fifteen minutes after being placed in this solution, the tension of oxygen in the blood is diminished from 19.1 to 3.7 per cent. This anoxemia was believed to be a result of injury to the gill membranes and to the change in the water and salt content of the blood with the attendant change in size and the destruction of the blood corpuscles, thus decreasing the gas carrying power of the blood.

However, if fish were dying from asphyxia due to an injury of the gill membranes while the irritability of the respiratory center remained normal, hyperemia and dyspnoea might be expected. They did not occur in *Squalus sucklii*. The changes observed correspond more with those to be expected from a depression of the respiratory center.

As a result of this investigation and the contributions to this problem made by others, the author is inclined to explain the death of fish in media having an abnormal salinity to a progressive depression of the respiratory center. Although a change in the composition of the surrounding medium frequently alters the permeability of the limiting membranes of the body, the changes in the osmotic pressure of the blood and the injury to the gills, provided they occur, are considered to be only a contributary cause of death. The action of the abnormal medium on the respiratory center and perhaps on the motor nerves supplying the gills appears to be the primary cause of death. Changes in permeability of the limiting membranes are of importance in this regard insofar as they alter the activity of the structures mentioned by (*a*) making it possible for salts to diffuse into or from the inner ear and brain cavity, (*b*) initiating a reflex over some cutaneous nervous structure (lateral line?), (*c*) altering the composition of the blood supplying these structures. The relative importance of these factors may, of course, be subject to variations. According to this concept, teleosts are more resistant to changes in salinity, either because the changes mentioned above occur less readily or because the fish are more resistant to the changes after they do occur. In support of mechanism (*a*) we may consider the suggestion of Loeb and Wasteneys (10) that toxic salts ($NaNO_3$, NaBr, KCl) in the external medium exert their action in this manner. Furthermore, it is to be expected that a disturbance of equilibrium might be concomitant with changes in the inner ear. This phenomenon has been observed shortly before death when fish were placed in solutions of abnormal salinity, Loeb and Wasteneys (10) and Young (14), in solutions of glycerine, Siedlecki (24), and in solutions of low oxygen and high carbon dioxide content, Wells (21). Maxwell (25) states that in most selachians the lymph of the vestibule is in free communication with the exterior sea water through the ductus endolymphaticus, and that it is reasonable to suppose that the density of the lymph would be practically equal to that of sea water. Since this condition is never encountered in teleosts it may be that we have here an important factor in the different susceptibility of these two groups of fish to variation in salinity.

Cardiac Movements.—Observations of the heart beat of *Squalus sucklii* were confined to fish in sea water, tap water and fish that were removed from one of the other experimental solutions following cessation of respiration. In general the heart is much more resistant to changes in the external media than is the respiration.

The heart rate of fish in sea water, taken shortly after exposure of the heart varied between 14 and 31 beats per minute with an average of 18.9.

In the experiments where records were made of the cardiac movements while the fish were in tap water very little change in the beat occurred. The fish were removed from the tap water at the end of an hour, or earlier in those cases where the respiration ceased in less than an hour. No failure of the cardiac movements occurred during this interval. No irregularities nor loss of tone of the heart muscle occurred other than those changes associated with the gill cleaning and struggling movements. A slight decrease in the amplitude of the contractions did occur and the number of beats per minute at the end of the hour varied between 4 and 28 with an average of 14.6.

Scott (3) records a heart rate of 50 per minute in a pithed specimen of *Mustelus canis* immersed in sea water. After the fish had been in fresh water for an hour the amplitude of the beat was about the same as at the beginning but the rate was only one fourth as great. When the respiration failed the heart beat was forcible and strong but it failed rapidly after this.

This investigator also records that in a pithed specimen of *Squalus acanthias* placed in the same medium from which it was captured (brackish water) the rate was 16 per minute. One specimen was placed in fresh water and observed for five and one half hours. At the end of this time, the heart rate was 8 per minute.

Scott (3) observed Traube-Herring waves in blood pressure records made from pithed specimens of *Mustelus* and thought that they might be the result of the destruction of the spinal cord. They ceased when the animal was placed in fresh water. Greene (12) observed somewhat similar waves in the tracings made from the Chinook salmon but considered them to result from the rhythmical effects of the respiration on the blood pressure. Lyon (17) observed something similar to Traube-Herring waves in blood pressure tracings made from sharks in sea water.

Rhythmical variations in cardiac tone (tonus waves) were observed in *Squalus sucklii* but they were more prevalent while the fish were in tap water than with fish in sea water. They appeared to begin and sometimes to end with the gill cleaning movements. Respiratory waves were not observed in the heart records. (See figures, page 177.)

Changes in the Amount of Hemoglobin.—We determined the amount of hemoglobin in 44 fish before and after immersion in the experimental solutions. Meischer's modification of Fleischl's hæmoglobinometer was employed in making these determinations. This apparatus was calibrated for human blood and since it was not checked against any other method, the absolute amount of hemoglobin recorded may not be correct. However, the same method was employed for all the determinations and the relationship between them is therefore of value.

The results obtained from normal fish varied between 0.68 and 5.14 grams of hemoglobin per 100 cc. of blood with an average value of 2.84. We were unable to demonstrate any definite relationship between sex or weight of fish and the amount of hemoglobin. In an examination of the blood of the skate, Harris (26) found that the percentage of HbO_2 in the blood of this animal varied between 3.1 per cent. and 6.2 per cent. He considers that the average value for the normal skate is between 3.5 per cent. and 3.8 per cent. By the use of Oliver's tintometer, v. Fleischl's hæmometer or the spectrocolorimetric method of Rollet he obtained results which were in close agreement.

The average changes in amount of hemoglobin shown in Table II. are in close agreement with the differences in osmotic pressure which the fish encountered when transferred to the abnormal media. They appear to result largely from the dilution or concentration of the blood which followed the change in osmotic pressure of the external medium. However, it has been observed by Hall, Grey and Lepkovsky (27) that in the menhaden asphyxia leads to an increase in the hemoglobin value. A similar change may have been operating in *Squalus sucklii.*

Changes in Weight.—When the impermeability of the gills is decreased as the result of introduction of a fish into a solution having a salinity to which it is unaccustomed, water and salts would enter or leave the blood and tissue of the fish depending on the direction of the difference in the osmotic pressure. The direction and extent of the exchange of water might be indicated by a study of the weights of fish before and after the change in the external media had been made. It has frequently been observed that teleosts gain in weight when in a medium having a lower osmotic pressure than normal and lose weight in a hypertonic solution, Greene (12), Portier and Duval (28).

However, it was found by Gueylard and Portier (29) that sticklebacks "unlike any other fish" gain in weight in hypertonic and lose weight in hypotonic solutions, while Sumner (30) and Scott (31) observed that fish frequently gained but later lost in weight in the same solution and Scott especially noted that the changes in weight varied greatly for individual fish.

Regarding the average changes in weight of *Squalus sucklii* recorded in Table II.: fish in distilled water, tap water and tap water with a pH of 8.4 made a gain in weight which may be ascribed to the greater osmotic pressure of the blood than of the environment. The fish in tap water gained 3.66 per cent., a result which agrees well with the observation of Scott (3) that the average gain in water in a number of the tissues of *Mustelus* after immersion in fresh water was 3.1 per cent. The fish in distilled water gained less than those in tap water a result which may be explained by the fact that the latter fish lived on an average of 40 minutes longer in the tap water than did the fish in distilled water. The fish in fresh water pH 8.4 made a much greater gain in weight than did the others although they remained in the medium only a few minutes longer than the fish in distilled water. The large gain may be taken as indicative of an extensive injury to the gill membranes by the alkaline solution. The osmotic pressure of the solution containing NaCl, $CaCl_2$, KCl and $MgSO_4$ would be about that of sea water. Practically no change in weight resulted in *Squalus* placed in this solution. The osmotic pressure of the solution of NaCl, $CaCl_2$, KCl; of NaCl, $CaCl_2$; and of NaCl is each progressively lower than sea water. A loss of weight was observed in fish placed in each of these solutions. Fish placed in sea water to which salts had been added gained in weight, although the osmotic pressure of this solution was greater than that of the blood.

In general, the length of time fish were able to live in a solution was not related to the change in the weight of the fish. These results as well as those of other investigators previously mentioned indicate that a study of the changes in weight gives results which are difficult to interpret. The amount of water and salts passing through the gill membranes and the amount of these substances eliminated by the kidneys should largely determine the change in weight of a fish placed in an abnormal medium. Marked individual variations of these two factors may explain the peculiar results noted.

Variation in Weights of Liver, Spleen and Pancreas with the Weight of the Fish.—The weight per gram of fish of the liver, the spleen and the pancreas taken from fish that had died in sea water varies with the weight of the fish. Although individual fish frequently show considerable variations in the relative weights of these organs, when an average is taken of the results from a number of fish with approximately the same body weight, a rather definite relationship becomes evident (Table III.).

In fish taken dead from sea water the average weight of the liver per gram of fish rises rather rapidly from 0.066 for fish weighing between 300 and 999 grams to 0.115 for fish with weights between 3,000 and 3,999 grams. As larger fish are considered, the figure tends to remain comparatively constant; that for fish weighing between 6,000 and 6,999 grams being 0.117.

With the spleen, the greatest weight per gram of fish (0.0040) occurs in fish with a body weight between 300 and 999 grams. In larger fish, the figure falls in a rather regular manner until with fish having a total weight between 6,000 and 6,999 grams, the weight of the spleen per gram of fish is 0.0015.

The pancreases taken from fish that had died in sea water show a variation similar to that noted in the case of the spleens. With fish whose weight lay between 300 and 999 grams, the weight of the pancreas per gram of fish was 0.00286, and for fish weighing between 6,000 and 6,999 grams, the figure is 0.00142.

Pregnancy or sex did not appear to result in an alteration in the weight of the organs and therefore in this connection, these factors may be neglected. Insufficient data, however, is at hand to permit the formulation of an opinion as to whether the increased weight of the fish due to the presence of embryos was balanced by a compensatory decrease in the weight of the body of the fish or an increase in the weight of the organs. Since the weights recorded were obtained from fish captured only during

TABLE III.

AVERAGE WEIGHT OF ORGANS PER GRAM OF FISH.

Organ.	From Fish that Died in	Fish with Body Weight between (gm.)							
		300–999	1,000–1,499	1,500–1,999	2,000–2,999	3,000–3,999	4,000–4,999	5,000–5,999	6,000–6,999
Liver	Sea water	0.0660^{44}	0.0769^{9}	0.0940^{6}	0.1104^{22}	0.1156^{8}	0.0984^{5}	0.1235^{6}	0.1176^{4}
	Distilled H_2O	0.06324^{16}			0.1213^{2}		0.103^{2}	0.0826^{2}	
	Tap water	0.0602^{9}	0.0756^{7}	0.113^{3}	0.114^{1}	0.124^{3}	0.0897^{7}	0.0824^{8}	
	Tap water pH 8.4				0.1114^{2}	0.134^{1}			
	NaCl solution	0.0740^{4}	0.0664^{1}		0.0904^{1}	0.0468^{1}	0.110^{1}	0.0814^{1}	
	NaCl, $CaCl_2$ solution			0.1027^{2}	0.1246^{1}	0.0984^{1}			
	NaCl, $CaCl_2$, KCl solution	0.0704^{5}			0.1140^{1}				0.0894^{1}
	NaCl, $CaCl_2$, KCl, $MgSO_4$ solution	0.0658^{2}	0.0556^{1}	0.0880^{1}	0.1155^{2}				
	Sea water with added salts	0.0479^{3}	0.0927^{1}	0.0637^{1}			0.0971^{1}		
Spleen	Sea water	0.00407^{44}	0.00416^{9}	0.00345^{6}	0.00275^{22}	0.00271^{8}	0.00203^{5}	0.00167^{6}	0.00153^{4}
	Distilled H_2O	0.00475^{16}	0.00418^{2}		0.00300^{2}		0.00289^{2}	0.00189^{2}	
	Tap water	0.00439^{9}	0.00365^{7}	0.00326^{3}	0.00311^{1}	0.00214^{3}	0.00244^{7}	0.00231^{8}	
	Tap water pH 8.4	0.00312^{1}			0.00325^{2}	0.00261^{1}			
	NaCl solution	0.00355^{4}	0.00606^{1}		0.00357^{1}	0.00276^{1}	0.00238^{1}	0.00206^{1}	
	NaCl, $CaCl_2$ solution			0.00349^{2}	0.00254^{1}	0.00189^{1}			
	NaCl, $CaCl_2$, KCl solution	0.00390^{5}			0.00232^{1}				0.00163^{1}
	NaCl, $CaCl_2$, KCl, $MgSO_4$ solution	0.00439^{2}	0.00324^{1}	0.00346^{1}	0.00296^{2}				
	Sea water with added salts	0.00374^{3}	0.00321^{1}	0.00300^{1}			0.00140^{1}		

TABLE III. (*continued*).

Organ.	From Fish that Died in	Fish with Body Weight between (gm.)							
		300–999	1,000–1,499	1,500–1,999	2,000–2,999	3,000–3,999	4,000–4,999	5,000–5,999	6,000–6,999
Pancreas	Sea water	0.00286^{44}	0.00253^{9}	0.00224^{6}	0.00199^{22}	0.00167^{8}	0.00170^{5}	0.00139^{6}	0.00142^{4}
	Distilled H_2O	0.00337^{16}	0.00248^{2}		0.00211^{2}		0.00173^{2}	0.00145^{2}	
	Tap water	0.00324^{8}	0.00240^{7}	0.00203^{3}	0.00296^{1}	0.00182^{3}	0.00155^{7}	0.00142^{8}	
	Tap water pH 8.4	0.00239^{1}			0.00266^{2}	0.00258^{1}			
	NaCl solution	0.00319^{4}	0.00305^{1}		0.00236^{2}	0.00136^{1}	0.00170^{1}	0.00165^{1}	
	NaCl, $CaCl_2$ solution			0.00266^{2}	0.00198^{1}	0.00150^{1}			
	NaCl, $CaCl_2$, KCl solution	0.00299^{5}			0.00171^{1}				0.00093^{1}
	NaCl, $CaCl_2$, KCl, $MgSO_4$ solution	0.00266^{1}	0.00244^{1}	0.00242^{1}	0.00185^{2}				
	Sea water with added salts	0.00243^{3}	0.00227^{1}	0.00242^{1}			0.00140^{1}		

A fish in tap water, weight between 7,000–7,999, weight per gram of fish; liver 0.103, spleen 0.00153, pancreas 0.00099.
Small figures indicate the number of fish used.

the summer months, nothing can be said regarding the occurrence of seasonal variations in the weights of the organs.

With fish that had died in the experimental solutions, the weight of the fish after cessation of respiration was used in calculating the weight per gram of fish for the organs examined. Although possible sources of error in the method of determining the weights of the organs per gram of fish may be pointed out, the methods used in determining these values for fish that had died in the experimental solutions are so nearly the same, that a comparison of the figures is certainly of value. In the case of fish dying in sea water and also in the case of fish dying in the experimental solutions the results would probably have been more uniform if a much larger series of fish had been available. In general the weights of organs taken from the two classes of fish were in close agreement. Weights of organs taken from fish that had died in distilled water usually did run *slightly* higher than those taken from fish that had died in sea water, while these latter weights in turn were usually higher than those obtained from fish that had died in sea water with added salts.

Gueylard (32) working with a fresh water teleost, the stickleback, found the normal weight of the liver to be 0.0435 grams per gram of fish but after a 24-hour sojourn in water containing 20 grams per liter of NaCl, the weight had decreased to 0.0420.

A marine fish such as *Squalus* might be expected to show an increase in the weight of the liver after being placed in distilled water and in tap water but the results obtained show that this is not the case.

It is the contention of Gueylard that a relationship exists between the weight of the spleen and the resistance of fish to changes in salinity. This investigator has found the weight of the spleen per gram of fish in four species of marine teleosts to average from 0.0005 to 0.00073, while the spleen of five species of fresh water teleosts ranges from 0.00065 to 0.0024. Since the figures for the dogfish which had died in sea water range from 0.00407 to 0.00153, this fish appears to have a weight of spleen corresponding to that of the fresh water teleosts.

In a study of the stickleback, a fish readily adaptable to changes in salinity, Gueylard (32) found the weight of the spleen per gram of fish to be 0.00535 while the average figure for five less adaptable species of fresh water teleosts was 0.00065–0.0027. She has further stated that if the stickleback were placed in water of its natural habitat to which 20 grams of NaCl per liter has been added, the weight of the spleen per gram of fish at the end of 15 minutes was 0.00356 and at the end of two hours 0.00266. Somewhat similar changes were observed when eels were transferred from fresh water to sea water (33).

Introduction of a marine fish into fresh water might therefore, be expected to produce an increase in the weight of the spleen. The fact that this change was not observed with *Squalus sucklii* may be taken as an indication that a relationship exists between the inability of the dogfish to make a change in the weight of the spleen and its lack of adaptability to changes in salinity. A second hypothesis is that in *Squalus*, the change in weight of the spleen occurs slowly and has not progressed sufficiently before death takes place to make itself apparent. Neither of these explanations appears satisfactory. Gueylard (32) also reports that when sticklebacks were placed in water to which NaCl had been added, the spleen became flabby and the color which normally was brownish red had changed to reddish yellow. No changes in the color or consistency were observed by the author when the spleens taken from dogfish that had died in sea water were compared with those taken from the experimental solutions.

It is a pleasure to express my appreciation to the Biological Board of Canada by whom a portion of the expenses incurred in this work has been defrayed. I am also grateful to Dr. W. A. Clemens of the Pacific Biological Station for the many facilities extended, to Professor A. W. Downs and Professor William Rowan of the University of Alberta for helpful suggestions, and to Viola W. Quigley for assistance with the practical work.

SUMMARY.

1. An investigation has been made of the effect on *Squalus sucklii* of transference to the following media: (*a*) distilled water, (*b*) tap water, (*c*) tap water pH 8.4, (*d*) NaCl solution, (*e*) NaCl, $CaCl_2$ solution, (*f*) NaCl, $CaCl_2$, KCl solution, (*g*) NaCl, $CaCl_2$, KCl, $MgSO_4$ solution, (*h*) sea water to which NaCl, $CaCl_2$, KCl, $MgSO_4$ had been added.

2. The most toxic solution was sea water with added salts, the solution of NaCl, $CaCl_2$ and KCl was the least toxic.

3. Tap water had a relatively low toxicity, but tap water which had been given the same pH as sea water by the addition of NaOH was more toxic.

4. Cessation of respiration invariably occurred more readily than did failure of the heart.

5. It appears likely that respiratory failure is the cause of death of fish in abnormal media. This is believed to result from a depression of the respiratory center.

6. No variation which could be ascribed to pregnancy, size or sex was observed in the duration of respiration or heart beat, the change in amount of hemoglobin, weight of fish or of organs while in the experimental solutions.

7. Changes in the amount of hemoglobin closely paralleled the changes in the osmotic pressure of the external media.

8. An increase in weight usually but not invariably resulted from introduction of fish into hypotonic media.

9. It could not be shown that a change in the comparative weight of the liver, spleen or pancreas followed the introduction of the fish into an abnormal medium.

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THE LUMINOUS MATERIAL OF *MICROSCOLEX PHOSPHOREUS* DUG.

STANISLAW SKOWRON.

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In my former paper ('26) I have described the luminescence of *Microscolex phosphoreus* and some of its chief characteristics, which seem to exclude the possibility of infection with luminous microörganisms in this species. The present note deals with the luminous material alone, and with its changes during the emission of light. The changes accompanying the light production have never been adequately studied in living forms, owing to technical difficulties or unsuitable material. The results of Hickling ('26) who investigated the luminous slime of a fish species, *Malacocephalus lævis*, differ in many points from my own observations on *Microscolex*.

The luminous slime of *Microscolex*,[1] usually discharged in small quantities and examined in a drop of tap water to keep it moist, consists of not very numerous small granules, which are situated within large, round cells, or set free into the surrounding fluid. Their round shape may be somewhat modified, owing to the close contact with other granules, and their diameter although very variable does not exceed a few microns. In many cases the granules form compact aggregates within the cells and show sometimes Brownian movement which does not cease at higher temperature of 80° C. and in 90 per cent. alcohol. They are of greenish color, strongly refract the light and do not show any optical activity. Under high magnification one can easily see that every granule is composed of two substances, one of which forms the central part, while the second lies around the periphery, an observation which appears to be similar to those of Förster ('12) on *Pholas* and of Hickling on *Malacocephalus*.

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[1] *Microscolex phosphoreus*, of rather common occurrence in Southern Europe, has never been as yet reported from Poland. Through the courtesy of Mr. Z. Wasyliszyn I received some specimens found in one of the coal-mines situated near Cracow. Visiting this place I observed in one passage not used for over two years, about 230 m. below the surface, great quantities of *Microscolex* which in these special conditions of constant temperature and moisture had propagated very rapidly. Walking in darkness, hundreds of luminous points were seen, glowing brilliantly after every step. This species seems to need a higher temperature and that explains why it can live in mines, where it was occasionally introduced.

Contrary to the individuals I have examined in Naples and others which were recently sent to me from Naples, the coal-mine forms do not show the day-night rhythm in luminescence, a fact probably connected with the constant darkness in which they are living. The degree of irritability of the nervous system, regulating the ejaculation of the luminous slime, seems to be the same during the night and day time, owing to these exceptional conditions.

The granules seem to be highly resistant to cytolytic reagents (chloroform, saponin, xylol, etc.) and do not change much after a long time. In hydrochloric acid, however, they swell to some extent and the central part of each granule dissolves at once, while the outer layer can be preserved for a few minutes without any visible changes. The next step is the slow dissolution of the latter. The granules are also quickly destroyed by pepsin solution at a certain pH and about 40° C. but the changes are markedly different from those produced by hydrochloric acid.

The picture of the slime if examined immediately after ejaculation and without any addition of water is very different. Besides the cells and granules mentioned above, there are great masses of large cells filled completely with granules, the other cells being already broken and the granules set free. If now a drop of water be put near the edge of the cover-glass, it is possible to observe how in the moment of contact with diffusing water the bright flash of light is produced and the granules dissolve instantaneously leaving sometimes but a very transparent sheath similar to the shadows of red blood corpuscles in hypotonic solution. During this process many cells are breaking up, but some of them remain intact and the water passing through the permeable cell membrane dissolves the granules situated in the cell. In this case one can see afterwards a fine network within the cell which represents the rest of the dissolved granules. As far as I was able to observe, the granules dissolving in water look quite as the formerly described ones, except that their size is usually greater and it seems to me that they are but fully developed stages of the same kind of structures. This is further corroborated by the fact that the undeveloped granules are never as numerous in the single cell as the others.

It is necessary to mention that every cell contains only undeveloped or developed granules alone, and in the slime one can always find both kinds of these cells, sometimes the cells with undeveloped granules being more numerous than usual but never as abundant as the others. Studying under the cover-glass the dissolution of the granules and the luminescence which lasts as long as granules are present, one must regard the dissolving granules as the real luminous material, a conclusion which disproves entirely the possibility of infection with luminous bacteria.

In order to find out the necessary conditions for luminescence I have first tried the effect of osmotic pressure of the surrounding medium, which, as pointed out by Hickling, plays a chief rôle in the diffusion of luminous substances from the granules of *Malacocephalus*. The experiments were, however, without result, as the luminous slime glowed in saturated KCl solution almost as bright as in distilled or tap water, the granules dissolving rapidly. Many similar observations showed that the osmotic pressure and different ions have no effect whatever on dissolution and luminescence of the luminous material. Glucose solutions gave different results. In higher concentrations both glucose and glycerin suppressed luminescence of the slime, which returned, however, upon addition of water. It seems that the amount of water regulates the dissolution of the granules and thereby the luminescence. Filter paper saturated with luminous slime ceases to glow in alcohol absolute and the granules remain intact, but the luminescence returns if some water is added. Taking for granted the importance of water, it will be easy to explain why the luminous slime, when ejaculated, glows longer in a moist atmosphere than in pure water, its quantity being the same in both cases. Larger drops of slime can be luminescent even in pure water for a few minutes because they form viscous clusters through which the water diffuses slowly.

I am not quite certain what prevents the luminescence of the granules inside the body of the worm. The amount of oxygen is ruled out of the question because the slime can be made luminous in water where the oxygen is in such small quantity that colorless reduced methylene blue does not turn blue. The amount of oxygen necessary for luminescence must be very small indeed, contrary to the observations on *Malacocephalus*, and without any doubt smaller than its supply in the cœlomic fluid of the living animal. Greater quantities are necessary for the ejaculation of the slime only, which function however is under control of the nervous system. The amount of water in the secreted fluid is also sufficient because the slime gives off the light for some time even in dryness, not as long however as to use up all the luminous material, and here lies the explanation why the dried worms or slime are still luminous upon moistening. The process of breaking up the cells always occurs when the luminescence proceeds and the amount of water is not too abundant, because in this case the dissolution of the granules may take place within the cell membrane. It seems to me that the changes occurring normally during or after the ejaculation of the slime in the cells themselves, probably in their cortical layer, lead to the contact of granules with surrounding water and this causes their luminescence.

As I have already pointed out, the highly concentrated glucose solution, alcohol abs., and also pure ether and chloroform stop the luminescence but the process is reversible if water is added and the action of these reagents is not too prolonged. Quite different is the effect of $HgCl_2$, concentrated solution or diluted to one half with water. The power of luminescence is in this case irreversibly destroyed. The same effect can be obtained with alcohol abs. acting for over half an hour.

The granules are luminescent at pH from 3 to 11. More acid or alkaline solution extinguishes the luminescence irreversibly. It was impossible to show quantitatively the duration of luminescence in relation to different temperatures, owing to the difficulty in getting exactly the same quantities of slime in all cases. Nevertheless it was found that the light is fainter and of shorter duration both in higher (40–50° C.) and lower (−5–10° C.) temperatures.

Discussion.

It seems more than probable that the two substances which compose the luminous granules are both necessary for luminescence. We may suppose that these two substances represent luciferin and luciferase separated from each other by a film as suggested by E. N. Harvey, while discussing the luminescence in other animals. When the cell breaks up and water is absorbed by the substances of the granules, the film may be destroyed and the fluid mixture glows till the whole material is used. It is possible that this destruction can be accelerated by the small addition of ether or chloroform to the water because of the effect of these solutions upon the intensity of luminescence, as I have formerly described. If the water is present in a very small quantity, the structure of many granules is not changed and the power of luminescence can be retained. The same result can be obtained when strong alcohol is added. Other substances, *e.g.*, $HgCl_2$, change chemically or physically the composition of the luminous material and these changes are irreversible. Osmotic pressure of the surrounding fluid does not play any rôle, contrary to the observations of Hickling on *Malacocephalus* who did not see the dissolution of the granules because he thinks that "in the artificial conditions of experiment the granules never become exhausted, since conditions automatically become unsuitable long before exhaustion occurs" owing to the too low pH for luminescence. In *Microscolex* the material was suitable enough to observe the dissolution of the granules and further investigations may show if this process is of more universal occurrence, because, as far as I know, it has only been mentioned by E. N. Harvey in jellyfishes by the action of saponin, sodium glycocholate and of fresh water. Besides, it is possible that different types of changes in luminous material may be met with, of which *Microscolex* and *Malacocephalus* are but two examples.

I am greatly indebted to Professor E. N. Harvey for criticizing my results.

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FUNCTIONLESS MALES IN TWO SPECIES OF *NEUROTERUS*.[1]

J. T. PATTERSON.

In connection with a proposed cytological investigation of the Cynipidæ, I have found it necessary to make a rather extensive collection and study of the different species present in the Austin area. Many new species and varieties have been discovered. These have been turned over to Dr. A. C. Kinsey for identification and description. Dr. Kinsey has already included a number of the Austin species in his important publications on the Cynipidæ, and others will be dealt with in future papers. I wish to take this opportunity to express my appreciation of his fine coöperation in this work.

During the course of this study many interesting facts concerning the biology of the Cynipidæ have been brought to light. Not the least among these is the discovery of functionless males in the (so-called) agamic generation of two species of *Neuroterus*. The species concerned are *N. contortus* (Weld) and *N. rileyi* (Bassett). Dr. Kinsey has recently redescribed these species and their varieties.

Neuroterus contortus has two generations a year, one called "*N. contortus* agamic form *contortus*," the other "*N. contortus* bisexual form *principalis*" (Kinsey, '23, pp. 91–92). Here at Austin this species is restricted to a single locality. Indeed, it is found in small clumps of sprouts around the base of two trees of *Quercus breviloba*. The galls of the agamic generation are found on the roots of the young shoots, just below the surface of the soil. The adults of this generation emerge during the last week of January and the first two weeks of February (1923). The female cynipid oviposits immediately in the tissues of the newly formed sprouts, just above the old galls, and usually above the level of the surface of the ground. By the middle of February the new galls begin to appear and are fully formed by the middle of March. The adults of the bisexual generation emerge during the first two weeks in April (April 3–15, 1922). New galls of the next agamic generation were first recognized on May 19, 1922.

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[1] From the Zoölogical Laboratory of the University of Texas. Contribution No. 209.

There is an interesting difference in the behavior of the females of the alternating generations. Those of the agamic generation react positively to light, while those of the bisexual generation react negatively. This was determined by tests in the laboratory, as well as by observations in the field. This difference in behavior may account for the relative positions (the agamic gall on the root, the bisexual on the stem) of the galls of the two generations.

I have bred out from the galls of the bisexual generation a total of 576 females and 1,076 males. This apparent disproportion of males to females is not due to any real difference in sex ratios, but is to be accounted for on an entirely different basis. I have found in this, and in certain other species of Cynipidæ, that all of the adults emerging from a single, isolated gall are of the same sex. This means that the agamic females must be of two kinds, one female- and the other male-egg-producing. If one collected galls a majority of which happened to contain adult wasps of one sex, this would give a larger number of individuals of that sex in the totals.

From galls of the agamic generation I bred out 231 females and 10 males. It is this interesting fact that forms the basis for the present note. I suspected from the first that these males might be functionless, and consequently decided to test out their sex behavior. On February 5, 1923, I bred out 62 females and 6 males from one lot of galls. As soon as they emerged the males were isolated and kept in a vial for several hours. Females were then placed with these males, and the culture kept under observation for an hour. During this period no matings took place. The females did not display any sex reactions, but some of the males did exhibit certain of the "courtship" reactions, such as approaching the female and vibrating the elevated wings. A single male made a very weak effort to mate with a female, but copulation did not occur. The experiment was repeated in the afternoon, but no attempts to mate were observed.

In order further to test out the behavior of these wasps, I collected a number of galls of the agamic generation and placed them in the ice box, on February 25, 1923. These were left there until the bisexual generation emerged in April. On April 14 the galls were taken from the ice box and kept at room temperature, and on the following day four agamic females came out. These were then tested with males of the bisexual generation. Before making the tests, however, the behavior of the males of the bisexual generation toward the females of that generation was studied. The normal sex reaction is as follows: As soon as virgin females are introduced into the dish containing the males, the latter become exceedingly active, and race rapidly about the dish. The male then approaches to within two or three millimeters of the female, stops, raises the wings above the body, and then rapidly vibrates the elevated wings. If the female responds, mating follows immediately. During this courtship on the part of the male, the female assumes a passive attitude, by becoming quiet, and then lowering the antennæ until the tips rest on the bottom of the dish. She remains perfectly motionless during the process of mating.

The four agamic females were introduced to a group of newly emerged males of the bisexual generation. The behavior of these males in the presence of the agamic females is identical with that described above. They show the normal sex reactions. On the other hand, these females do not give the slightest evidence of response. In fact, they make every effort to escape from the aggressive males. During the course of an hour about fifty attempts were made by the males to mate with these females, but there was not a single successful copulation. It would have been interesting to study the behavior of these males in the presence of the sexually active females of the bisexual generation, but unfortunately no males emerged from the refrigerated material.

It is evident from these observations that the agamic females have entirely lost the mating instinct. It is also clear that the aberrant males have nearly lost this same instinct and, so far as reproduction is concerned, are functionless.

The second species in which similar functionless males have been found is *Neuroterus rileyi* variety *mutatus* (Kinsey, 1923, p. 118). Only the agamic generation of this variety is known. It produces an enlargement of the stems of *Quercus Muhlenbergii*. From April 4 to 11, 1922, I reared 94 females and 3 males from three galls. The sex behavior of these males is similar to but weaker than that of the functionless males of *contortus*.

The presence of a few aberrant males in the agamic generation of species showing the typical alternation of generations is of considerable interest, because it indicates that such males represent a remnant of a more primitive, bisexual condition in the generation which has now become agamic. As Kinsey points out, these observations are of considerable importance in connection with our understanding of the development of agamy and heterogeny in the Cynipidæ. I have no doubt that careful observations will reveal the presence of similar males in the agamic generation of other species. In most species, however, such males are evidently not present.

Another point of interest is the one concerning the origin of these males. Our cytological studies indicate that males of all species of the Cynipidæ are haploid, while the females are diploid. Males arise from unfertilized eggs that have undergone chromatin reduction. The females of the bisexual generation come from eggs which have failed to reduce the number of chromosomes, while those of the agamic generation develop from fertilized eggs. I have accumulated evidence which offers a possible explanation of the fact that the bisexual generation produces an agamic generation of females. In several species examined, the sexual females usually do not oviposit until after fertilization takes place. Eggs laid by these females should be fertilized and consequently would produce females. The few functionless males appearing in this generation might arise in one of two ways: either certain unimpregnated females occasionally lay eggs that develop into males or else an inseminated female lays a few unfertilized eggs, after the manner of the queen bee. Such eggs would produce males.

The suggestion that certain males are functionless in the Cynipidæ is not entirely new. Thus Adler ('81) has pointed out that in the genus "Rhodites" the few males found in certain species must be superfluous, but so far as I am aware the two cases cited above are the first to be recorded for the agamic generation of heterogenous species.

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BIOLOGICAL BULLETIN

SEXES IN THE CYNIPIDÆ AND MALE-PRODUCING AND FEMALE-PRODUCING LINES.[1]

J. T. PATTERSON.

I. Introduction.

In certain groups of insects there occurs an alternation of generations in which a bisexual generation alternates with one or more parthenogenetic or agamic generations. Thus in Aphids and Phylloxerans such an alternation occurs, and in many of the gall wasps, or Cynipidæ, a similar condition is found. In the case of Phylloxerans, Morgan has pointed out that in some species (*e.g.*, *P. fallax*) a single agamic female lays (probably) both male-producing and female-producing eggs, while in other species (*P. caryæcaulis*) a given agamic female lays but one kind of egg. Her progeny therefore will be either all females or else all males. I have found similar conditions to exist among the Cynipidæ, and it is the purpose of this paper to give the essential facts.

The cytological basis of sex-determination in the Aphids and Phylloxerans is very well worked out, but the same can not be said for the Cynipidæ. However, the facts already known are sufficient to indicate that the males of all species are haploid, while all females are diploid. Males develop from unfertilized eggs in which the number of chromosomes has been reduced during maturation. Females develop either from fertilized eggs or from unfertilized eggs in which reduction has not taken place (Doncaster).

This means that the primitive species, which usually have a single bisexual generation a year, develop by facultative parthenogenesis. In such species all eggs are alike and all undergo the typical maturation divisions with reduction. If the egg is fertilized it produces a female (diploid), but if unfertilized it develops into a male (haploid). It is not known whether the unimpregnated female can lay both fertilized and unfertilized eggs, as is the case in the queen bee, or whether the unimpregnated female alone lays unfertilized eggs.

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[1] Contributions from the Zoölogical Laboratory, University of Texas, No. 210.

In species showing alternating generations the agamic females are the product of the fertilized eggs of the bisexual generation, but all of the individuals, both males and females, of this generation develop from unfertilized eggs laid by agamic females. As we shall show, in some species the female lays both male-producing and female-producing eggs, while in other species a given female lays but one kind of egg. Finally, in some of the more highly specialized Cynipidæ males are never found, and development is exclusively by parthenogenesis.

In breeding out various species of Cynipidæ for other purposes, a large number of data on sex ratios has incidentally been accumulated. From this we may select such cases as have a bearing on the questions outlined above. The plan has been followed of collecting single, isolated galls and breeding out all of the individuals from each gall. In this way it is possible largely to eliminate the danger of breeding from a gall which has been produced from the eggs of two or more females.

2. Sexes in the Primitive Genera.

Two genera of the primitive Cynipidæ have been investigated here—the genera *Aulacidea* and *Aylax*. Two species belonging to the first genus have been bred out. Both of these species infest the wild lettuce, *Lactuca ludoviciana*. These are *Aulacidea bicolor radialis* and *A. tumida conjuncta* (Kinsey, MS.).[2] *Bicolor* does not produce a gall but inhabits the pith of the stem. *Tumida* produces a distinct gall on the lettuce, usually in the form of a twisted swelling at the top of the stalk.

I have reared 20 females and 15 males of *A. bicolor*, and since it is one of the most primitive species, males and females are expected to appear in about equal numbers.

I have bred out 231 individuals from the galls of *A. tumida.*

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[2] I am indebted to Dr. A. C. Kinsey for the identification or description of the species of Cynipidæ mentioned in this paper.

Of this number 51 only, or about 23 per cent., are males. In breeding from single isolated galls, it is found that each gall yields both sexes (Table I.).

TABLE I.

Aulacidea tumida conjuncta.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	11	1	D	2	2
B	6	1	*E*	5	1
C	5	4	Mass	151	42
Totals	180	51	Per cent. of males, 22.51		

The genus *Aylax* is represented by a single species, *Aylax laciniata compacta* (Kinsey, MS.). It produces clusters of galls on the receptacles of the flowers of the rosinweed, *Silphium albiflorum*. The galls are formed in the sterile flowers toward the center of the disc. A total of 405 individuals of this variety have been bred out. About 45 per cent. of these are males, indicating that the sexes occur in about equal numbers.

TABLE II.

Aylax laciniata compacta.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	2	3	*G*	2	0
B	2	0	*H*	4	1
C	4	0	*I*	7	0
D	3	0	*J*	2	2
E	5	3	*K*	1	1
F	2	4	Mass	192	165
Totals	226	179	Per cent. of males, 44.19		

The rearings from isolated galls are shown in Table II. Of the eleven galls bred from, six yielded individuals of both sexes and five those of one sex. On account of the numerous parasites, it is difficult to secure more than a few cynipids from each flower head. Thus 64 parasites and only 48 cynipids were bred out from the eleven galls.

The genus *Rhodites* is generally regarded as one of the primitive, in that none of its species is known to have an alternating generation. However, in some respects it gives evidence of high specialization, especially in its concentration on the genus *Rosa*. Its interest in this connection is found in the fact that in many species the males are much fewer in numbers than the females. In certain species the males do not exceed two per cent., and are, according to Adler, superfluous. Kinsey ('20) believes that on the whole most of the species reproduce agamically, "with the males still existent but not usually abundant enough to fertilize many, if any, of the females." I have not been able to find any species of *Rhodites* at Austin.

3. Sex Ratios in Species with Alternating Generations.

Among the most primitive species with alternating generations are those found in the genus *Neuroterus*, but there are several other genera exhibiting the same condition. The few cases given below are selected with the view of bringing out the main points concerning the female- and male-producing lines of the agamic females.

a. Andricus operator austrior form austrior Kinsey.

This variety has been described by Dr. A. C. Kinsey ('22) from material collected here at Austin. It is a bisexual generation, and what is in all probability the alternate generation is an acorn gall on *Quercus marylandica*. From the acorn galls I have bred out six females. The bisexual galls occur on *Q. schneckii*. The gall is a large, compact mass of wool, within which are found clusters of seed-like capsules containing the larval cells. It involves the young stems, young leaf clusters, and flower clusters.

A total of 1,235 individuals have been reared. About 46 per cent. of these are males. The results of the breeding experiments are shown in Table III. The point of chief interest is the fact that a large majority of the galls yielded individuals of both sexes. Thirty-four of the forty-five galls bred from gave cynipids of mixed sexes, eight yielded females only, and three gave males only. There is no doubt that the majority of agamic females are capable of laying both male-producing and female-producing eggs. However, some of them lay but one kind of egg (Table III., galls *i–s*).

TABLE III.

Andricus operator austrior form austrior.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	2	4	*X*	2	1
B	25	4	*Y*	2	2
C	103	15	*Z*	8	3
D	69	33	*a*	18	2
E	16	8	*b*	2	1
F	4	2	*c*	21	1
G	21	6	*d*	15	1
H	11	2	*e*	17	5
I	4	2	*f*	5	2
J	71	2	*g*	10	1
K	26	8	*h*	2	1
L	5	4	*i*	8	0
M	34	11	*j*	3	0
N	35	1	*k*	5	0
O	2	3	*l*	15	0
P	8	1	*m*	2	0
Q	4	1	*n*	7	0
R	8	2	*o*	21	0
S	26	4	*p*	4	0
T	3	1	*q*	0	51
U	2	2	*r*	0	118
V	2	1	*s*	0	138
W	4	2	Mass	19	146
Totals	671	564	Per cent. of males, 45.66		

b. Andricus maxwelli Bassett.

This species forms polythalamous galls at the ends of twigs of *Quercus stellata*. It is an ideal gall from which to test out the kind of eggs laid by the cynipid. The galls are scattered and frequently a single gall will be found on one tree. Under such conditions it is highly improbable that a single isolated gall would be the product of the layings of more than one female. The number of individuals emerging from a single gall varies from one to as high as 27.

I reared 474 individuals of this species. About forty-two per cent. of these are males. The results of the breeding tests are tabulated in Table IV. Sixteen galls yielded males, eighteen yielded females, and seven gave individuals of both sexes. As compared with the preceding, *maxwelli* shows a distinct increase in the number of agamic females which lay one kind of egg.

TABLE IV.

Andricus maxwelli.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	4	23	*V*	6	0
B	13	14	*W*	2	0
C	4	7	*X*	4	0
D	11	4	*Y*	3	0
E	3	1	*Z*	0	12
F	1	3	*a*	0	4
G	1	1	*b*	0	1
H	1	0	*c*	0	5
I	25	0	*d*	0	4
J	10	0	*e*	0	22
K	20	0	*f*	0	15
L	8	0	*g*	0	11
M	14	0	*h*	0	4
N	6	0	*i*	0	2
O	8	0	*j*	0	4
P	25	0	*k*	0	9
Q	8	0	*l*	0	5
R	8	0	*m*	0	2
S	7	0	*n*	0	3
T	4	0	*o*	0	2
U	13	0	Mass	68	39
Totals	277	197	Per cent. of males, 41.56		

c. Andricus maxwelli var.

A distinct variety of the preceding makes a somewhat similar gall on the twigs of *Q. breviloba.* This variety has not as yet been described. It is No. 47 of my collection of Cynipidæ. I have been able to breed out 138 wasps of this species. The results are shown in Table V. Of the fifteen individual galls bred from, all but two gave individuals of one sex. This variety, therefore,

TABLE V.

Andricus maxwelli (variety).

Galls.	Females	Males.	Galls.	Females.	Males.
A	4	5	*I*	0	8
B	2	1	*J*	0	5
C	8	0	*K*	0	13
D	11	0	*L*	0	8
E	3	0	M	0	4
F	2	0	*N*	0	2
G	3	0	*O*	0	2
H	0	7	*Mass*	7	43
Totals	40	98	Per cent. of males, 71.01		

shows a further tendency for the agamic female to lay but one kind of egg.

d. *Eumayria floridana texana* (*Kinsey, MS.*).

This species produces a polythalamous gall at the end of roots of *Quercus schneckii.* Table VI. gives the results of the rearing tests. Of the 1,135 individuals bred out, 729 or 64 per cent. are males. Two of the eight isolated galls give both sexes, one yielding 124 females and a single male, the other, 8 females and 88 males. Each of the other six galls gave individuals of one sex. This species must have an agamic generation, for it has been observed that the females begin to oviposit in the leaves of the same tree immediately after emerging.

TABLE VI.

Eumayria floridana texana.

Galls.	Females.	Males.	Galls.	Females.	Males.
A..........	124	1	*F*..........	0	69
B..........	8	88	*G*..........	0	53
C..........	267	0	*H*..........	0	6
D..........	0	138	Mass	7 ·	225
E..........	0	149	Totals......	406	729
Total reared, 1135			Per cent. of males, 64.22		

e. *Belenocnema treatæ kinseyi form parallela* (*Kinsey, MS.*).

We may now consider four species in which the individuals emerging from one gall are always of the same sex. This means that the agamic female must be either a male-producer or a female-producer.

The polythalamous galls of the bisexual generation of *Belenocnema treatæ* variety *kinseyi* occur on the rootlets of the common live oak, *Q. virginiana.* The galls of the agamic generation (*B. treatæ kinseyi* form *kinseyi*) are monothalamous and are found on the under side of the leaves of the same oak. The galls of the bisexual generation invariably yield cynipids of the same sex (Table VII.). The sexes occur in about equal numbers. Of the 677 individuals reared, 329 are males. This gives about 49 per cent. of males.

Table VII.

Belenocnema treatæ kinseyi form parallela.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	7	0	*K*	0	7
B	0	8	*L*	5	0
C	0	33	*M*	6	0
D	10	0	*N*	22	0
E	2	0	*O*	8	0
G	7	0	*P*	23	0
H	0	16	*Q*	9	0
I	8	0	Mass	241	253
J	0	12	Totals	348	329
Total reared, 677			Per cent. of males, 48.59		

f. Neuroterus irregularis albipleuræ Kinsey.

This variety of *Neuroterus* forms galls on the young leaves of *Q. breviloba*. It is a typical bisexual generation, but the alternate or agamic generation is not known. The individual galls always yield wasps of one sex (Table VIII.). The sexes probably occur in equal numbers, although but 40 per cent. of the 902 wasps bred out are males.

Table VIII.

Neuroterus irregularis albipleuræ.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	0	20	*J*	18	0
B	0	19	*K*	7	0
C	0	9	*L*	0	3
D	5	0	*M*	0	12
E	0	9	*N*	0	4
F	10	0	*O*	4	0
G	41	0	*P*	2	0
H	0	8	Mass	454	243
I	0	34	Totals	541	361
Total reared, 902			Per cent. of males, 40.02		

g. Neuroterus vernus evanescens Kinsey.

This variety has galls in the form of swellings on the ament stem of *Quercus breviloba*. The results of the rearings are seen in Table IX. Perhaps not enough galls were bred out to show conclusively that individuals of one sex always emerge from a single gall. However, in addition to the five galls listed in the table, my field notes state that mixed sexes have never been seen emerging from one gall.

TABLE IX.

Neuroterus vernus evanescens.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	1	0	*D*	0	4
B	0	5	*E*	3	0
C	0	4	Mass	44	46
Totals	48	59	Per cent. of males, 55.14		

h. Plagiotrochus candidus (*Kinsey MS.*).

This species forms galls on the leaf petiole of *Quercus schneckii.* As a rule, a single cynipid emerges from one gall, but whenever there are two or more, they are of the same sex (Table X.).

TABLE X.

Plagiotrochus candidus.

Galls.	Females.	Males.	Galls.	Females.	Males.
A	2	0	*J*	1	0
B	1	0	*K*	0	2
C	3	0	*L*	1	0
D	1	0	*M*	1	0
E	1	0	*N*	4	0
F	2	0	*O*	1	0
G	0	1	*P*	1	0
H	0	1	Mass	11	72
I	3	0	Totals	33	76
Total reared, 109			Per cent. of males, 69.72		

i. Neuroterus niger pattersoni form pattersoni (*Kinsey*).

In all of the forms considered above the galls are polythalamous, or else clusters. It is interesting to know that in a species producing scattered monothalamous galls it is possible to test out the agamic female as to the kind of egg she lays. The opportunity to do this is found in *Neuroterus niger pattersoni.* Both generations are known. The galls of the agamic generation are found as small swellings on the leaves of *Q. breviloba*, and probably *Q. stellata* also, while those of the bisexual generation occur on the leaves of *Q. stellata.* The agamic females emerge in March and immediately oviposit their eggs on the underside of the post oak leaves. It was noted that once a female began laying her eggs in a given leaf, she never moved to another leaf, but continued until all of her eggs were laid. This suggested the possibility of breeding out the bisexual individuals from separate leaves. The results of these tests are shown in Table XI. Of the 26 leaves thus tested, 22 gave cynipids of one sex, and four gave mixed sex (leaves *W–Z*).

Table XI.

Neuroterus niger pattersoni form pattersoni.

Leaf.	Females.	Males.	Leaf.	Females.	Males.
A	55	0	*O*	52	0
B	31	0	*P*	0	9
C	0	19	*Q*	0	9
D	14	0	*R*	0	10
E	0	21	*S*	0	12
F	0	9	*T*	0	24
G	0	6	*U*	0	4
H	4	0	*V*	0	2
I	0	16	*W*	9	58
J	4	0	*X*	12	1
K	0	11	*Y*	8	53
L	0	19	*Z*	1	21
M	0	6	Mass	468	465
N	21	0	Totals	683	775
Total reared, 1,458			Per cent. of males, 53.15		

It is highly probable that the four exceptions are due to the fact that more than one agamic female laid in each leaf. That this is so, is indicated by breeding out ten galls recently. These galls were carefully selected from a region in which the galls were very scarce. Care was taken to secure a single isolated leaf from each of ten trees. Each of these ten leaves gave wasps of one sex (leaves *M–V*, Table XI.). From this it may be concluded that the agamic female of this species lays but one kind of egg.

4. Discussion.

The species listed above are a few of a rather large number in which the breeding tests indicate that the agamic females are of two types. The evidence shows clearly that among heterogenous species the function of laying one kind of egg by the agamic female has evolved from a condition in which such a female laid both male- and female-producing eggs.

The chief point of interest is whether it is possible to find cytological evidence that would be adequate to explain this condition in the Cynipidæ. Doncaster ('16) has attempted to solve the question of the nature of the difference between the female-producing and male-producing females of the agamic generation of *Neuroterus lenticularis*, one of the heterogenous species found in Europe. He attacked the problem both by breeding experiments and by cytological investigations. He concludes from the results of his breeding tests that the two types of agamic females could not be due to a dimorphism of sperms produced by one male. He suggests two other possibilities: (1) That the two types of female may be due to two kinds of eggs laid by different sexual females; (2) or, if each sexual female mates with only one male, they may be due to two kinds of males which produce different spermatozoa.

Doncaster found no evidence in the spermatogenesis which would support the last suggestion. In studying the maturation of the eggs laid by the sexual females, he did find differences indicating that there might be two classes of eggs. However, he did not regard these differences sufficient "to correlate them with the sex-phenomena with any confidence." The question of the nature of the difference between the two types of agamic females in the Cynipidæ is therefore unanswered, and its solution awaits further study.

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FACTORS INFLUENCING THE RATE OF METABOLISM OF *ÆSHNA UMBROSA* NYMPHS.

MARY HONORA SAYLE.

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INTRODUCTION.

The respiratory exchange of an animal is of special physiological interest, because it affords a means of securing quantitative evidence regarding the metabolic processes taking place inside the living animal. It may be measured by ascertaining either the oxygen consumption or the carbon dioxide output. At present, methods for the determination of the latter have been greatly improved and are especially favorable for work on lower forms, such as insects. The literature dealing with metabolic rate as influenced by feeding, starvation and change of temperature and light, includes a number of papers on the metabolism of insects, but little or nothing in the way of direct quantitative measurements of metabolism in dragon fly nymphs.

HISTORICAL.

Spallanzani (22) and Plateau (18) determined the vital limit of tolerance to high temperature of many species of insects. Spallanzani (23), Treviranus (25), von Linden (27), Weinland (28), and Fink (9) studied the gaseous exchange of various insects. Newport (15) and Sosnowski (21) determined the carbon dioxide output of insects in various stages of development. In the classical work of Regnault and Reiset (19), the carbon dioxide output of May-beetles was determined. Bütschli (6) and Vernon (26) studied the effect of temperature upon the gaseous exchange of *Blatta orientalis* fed on different substances. Slowtzoff (20), Batelli and Stern (2), Parhon (17), and Dirken (7) also observed the influence of temperature on the respiratory exchange of such insects as ants, bees, bettles, silkworms, and flies. Many experiments have been performed on the gaseous exchange of the silkworm. Of these the most outstanding ones are those of Luciani and Piutte (14), Luciani and Lo Monaco (13), and Farkas (8).

Loeb and Northrop (12), Bodine and Orr (5), and Northrop (16) studied the respiratory metabolism of *Drosophila* cultures. Bodine (3) (4) studied the water content and the rate of metabolism of active and hibernating grasshoppers. Studies on the influence of light on the metabolic rate were made by Loeb (11) on the chrysalids of certain butterflies and moths and by Allee and Stein (1) on the May-fly nymphs.

Perhaps the most useful compilation of data on metabolism is to be found in Krogh's (10) monograph on "The Respiratory Exchange of Animals and Man." This work includes the results of several original experiments on insect pupæ, particularly on chrysalids of *Tenebrio molitor*.

The purpose of the present study was to determine the effect of starvation, darkness, and temperature upon the carbon dioxide output of *Æshna umbrosa* nymphs. These nymphs are easily obtained and live successfully under general laboratory conditions. They offer especially desirable material for the study of the respiration in a truly aquatic form. Acknowledgment is made to Prof. A. S. Pearse for suggesting the problem and to Prof. L. E. Noland and Dr. Samuel Lepkovsky for advice and criticism during the course of the work.

MATERIAL AND METHODS.

The insects studied were the nymphs of *Æshna umbrosa* E. Walker, and were collected during September 1925 and 1926 in Lake Forest Creek, near Madison, Wis. The material used was collected at the same time and from the same place and hence was all of approximately the same age and had been under fairly uniform environmental conditions. The creek is a spring-fed, woodland stream about two feet wide and varying in depth from two inches to two feet. The stream is choked here and there with water cress to which the nymphs cling. The pH of the water on collecting days was around 8.

When the nymphs were not being used for experimentation, they were kept separately in finger bowls containing lake water, the pH of which ranged around 8.6. A bit of water plant was placed in the bowl with the insect, except in the case of insects subjected to starvation. These were supplied with a pebble on which to cling. The temperature of the water ranged from 20° to 22° C. The nymphs were fed pieces of mealworms three times a week and the water changed an equal number of times. Careful controls were run. When the nymphs were fed and changed to fresh water, the controls were also fed and changed. The nymphs used in the study were of about the same age, usually in the penultimate stage at the start of the experimental work. Three sets of ten insects each were used in each type of experiment and an effort was made to arrange the insects so that the weight of each set was about the same.

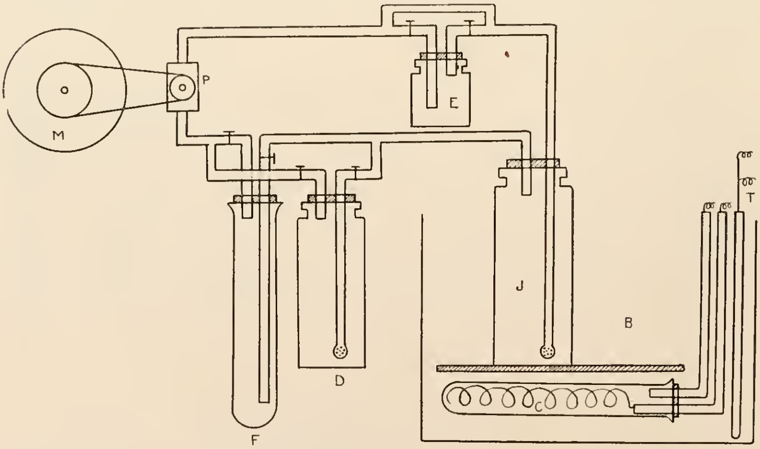


Fig. 1

The experimental apparatus consisted of a closed system of chambers and tubes, through which a circulation of air was maintained by means of a small air pump operated by an electric motor (Fig. 1, *M* and *P*). The airtight glass jar (*J*) in which the animals were placed, rested in a constant temperature bath (*B*), which was heated by an electric coil (*C*) with thermostat control (*T*). To provide constancy of illumination, light was supplied, when required in the experiment, by a 75-watt electric light bulb which was hung three feet above the jar.

At the beginning of each experiment, 300 cc. of physiological salt solution was placed in the jar. This solution consisted of 7 gr. NaCl, .3 gr. KCl, and .25 gr. $CaCl_2$ per liter of glass distilled water or a total of 7,550 parts per million of dissolved salts. The solution had a specific gravity of 1.003 at 24° C. and a pH near 7.4. Preliminary experiments showed that distilled water, which would naturally be preferred, was quite unsuitable; the nymphs died after a five-hour stay in the water. That the injurious effect of the distilled water was of an osmotic nature was shown by the fact that the addition to the same water of the neutral salts mentioned above made it a satisfactory medium.

In each experiment, a set of ten animals was used and each nymph was placed in a paraffin-dipped wire cage, just large enough to hold the nymph, so that body movement was reduced to a great extent. The cages were closed and tied together in sets of five, with a long thread attached to each set. Experiments were done at the same time each day and always forty-eight hours after feeding, so that excreta had been passed. At the beginning of each experiment, the nymphs were hung above the liquid in the clamp top jar and the threads issued from under the jar cover. The insects were placed in the jar before the CO_2 was removed from the circuit in order to avoid opening the jar after the preparatory run.

After the nymphs were thus arranged in the jar, the motor was started and the air was forced from this jar through a bottle (*D*) containing a solution of sodium hydroxide, which absorbed the carbon dioxide from the circuit and also from the liquid in the experimental jar. A test bottle (*E*) containing a solution of barium hydroxide was then opened into the circuit to determine whether or not all the carbon dioxide had been removed. Fifteen minutes were found to be sufficient time to remove the carbon dioxide. At the end of this time, the clamp top on the experiment jar was loosened slightly for a moment, releasing the string attached to the cages and allowing them to slip down, immersing the nymphs in the liquid. Bottles *D* and *E* were then cut off from the circuit by the glass stopcock and the experiment was actually started. The air in the circuit was bubbled through the Pyrex test tube (*F*) containing 25 cc. of $N/20$ $Ba(OH)_2$ for five hours. At the end of this period, the clamp top was loosened momentarily and the cages were drawn up to a point above the liquid, and then for fifteen minutes more the air was circulated to remove any carbon dioxide not already absorbed from the water.

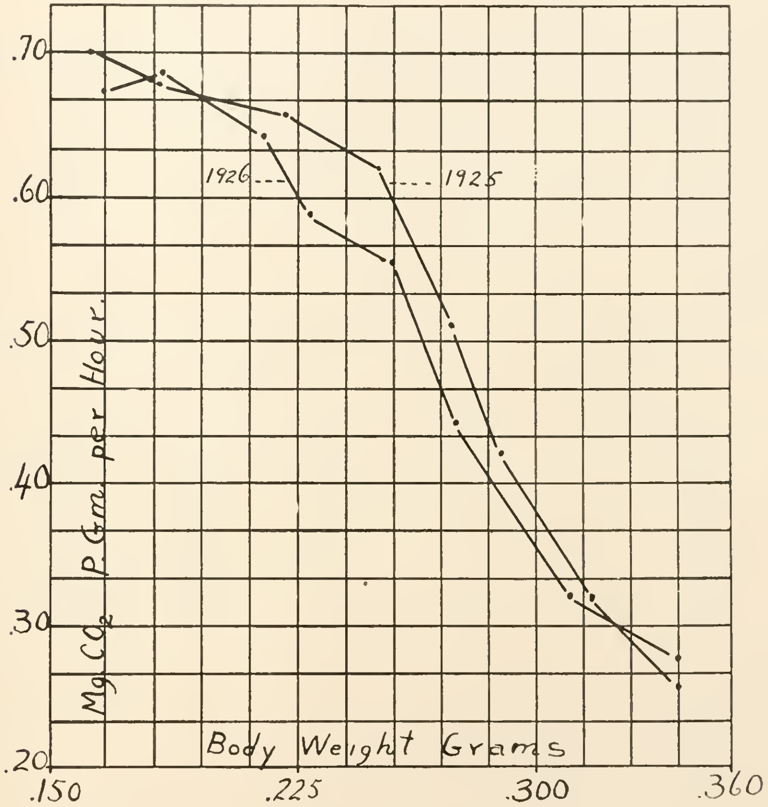
The test tube containing the carbon dioxide precipitated as barium carbonate was detached from the circuit and carefully protected from air until titrated. After the precipitate had settled, the excess of barium hydroxide was titrated with $N/20$ HCl, using phenolphthalein as indicator. To check the accuracy of titration, two aliquot portions (5 cc.) of the clear supernatant liquid were used and the average of the two, multiplied by five, was taken as the true value. This amount was then subtracted from the amount of $N/20$ HCl required to neutralize 25 cc. of the original barium hydroxide solution and the difference was taken as equivalent to the quantity of carbon dioxide produced by the organisms during the period of the experiment. This method of CO_2 determination is based upon the assumption that the only volatile acid produced by the nymphs is carbon dioxide, or that other acids, if produced, are negligible in quantity.

Since the rates of respiratory metabolism are expressed per gram of body weight per unit of time, it was necessary to weigh the insects used in the experiments. Weighing excited the insects greatly, so the process was performed immediately following rather than immediately preceding each experiment. Since the nymphs live in the water and alternately draw water into the rectum and expel it in the respiratory act, it was necessary to remove surplus water from the surface of the body and to see that the nymphs discharged water from the rectum before each weighing. The excess moisture was removed by placing the nymphs between pieces of filter paper. Even though the greatest care was used, it is probable that the amount of moisture remaining in the rectum varied a little at different weighings.

OBSERVATIONS.

A. The Carbon Dioxide Output of Control Insects.

The carbon dioxide output of the nymphs under ordinary laboratory conditions was taken as representing the standard output, to which comparison could be made with the data secured under experimental conditions. The temperature of the water was uniformly between 20° and 22° C. Two sets of insects were used each year (1925 and 1926) and the records of their CO_2 output are given in Table I., and the average record in Graph 1.



GRAPH 1. Curves show rate of CO_2 output over period of 3 months. Time interval 10 days. Temperature 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

TABLE I.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING A PERIOD OF THREE MONTHS AT 22° C.
Time interval—10 days.

1925.

Weight of 1 Insect in Grams.			CO_2 Output in Milligrams per Gram Body Weight.		
Set 1.	Set 2.	Average.	Set 1.	Set 2.	Average.
.160	.171	.165	.6630	.7391	.7010
.185	.183	.184	.6950	.6505	.6727
.220	.214	.217	.6501	.6499	.6500
.250	.238	.244	.6402	.5989	.6195
.274	.260	.267	.5218	.5076	.5147
.293	.288	.290	.4129	.4333	.4231
.319	.313	.316	.3343	.3101	.3222
.346	.334	.340	.2697	.2454	.2575

1926.

Weight of 1 Insect in Grams.			CO_2 Output in Milligrams per Gram Body Weight.		
Set 1.	Set 2.	Average.	Set 1.	Set 2.	Average.
.172	.166	.169	.6555	.6900	.6727
.191	.183	.187	.6968	.6731	.6849
.217	.214	.215	.6336	.6504	.6420
.237	.230	.233	.5737	.5903	.5820
.267	.255	.261	.5482	.5545	.5513
.291	.279	.285	.4350	.4791	.4570
.328	.301	.314	.3018	.3508	.3263
.359	.331	.345	.2757	.2884	.2820

The determinations were made every ten days and the data taken over a period of almost three months. The animals were still in good condition at the end of this period.

The results show a relatively smooth curve; the rate of CO_2 output is higher for animals lighter in weight and decreases progressively as the animals increase in body weight. Differences in body weight, especially in nymphs, are closely correlated with differences in age and one is led to assume that younger individuals have the higher rate of respiratory output. Smaller animals are generally more active and are immature and growing.

At 22° C. the saturation point of oxygen at the stated degree of salinity of the chloride solution is about 8. Since each experiment was run over a period of five hours, a determination of the dissolved oxygen content of the water at the beginning and at the end of one of the experiments was made, using the Winkler method as described in "Standard Methods for the Examination of Water and Sewage" (24). The physiological salt solution was found to contain 6.959 parts per million at the start of the experiment and 7.145 parts after five hours. It was thus shown that there was plenty of oxygen in the circuit to meet the needs of the insects for the period of the experiment. The fact that there is more dissolved oxygen in the solution at the end of this experiment than at the beginning is accounted for by the fact that the oxygen of the air in the circuit previous to starting the experiment dissolved in the solution during the five hours of circulation.

B. The Effect of Starvation on the Carbon Dioxide Output.

Three sets of nymphs were used in the starvation experiments, carried on at 22° C. Their carbon dioxide output in a well-fed condition was determined twice at the beginning of the experiment, and then the animals entered starvation. The water was changed regularly, but no food was given. Carbon dioxide determinations were made once a week up to the fifth week. In each set, one or more animals died during the fifth week, which necessarily ended the experiment.

An examination of Table II. and Graph 2 shows that the carbon dioxide output decreased during the first week, but in each

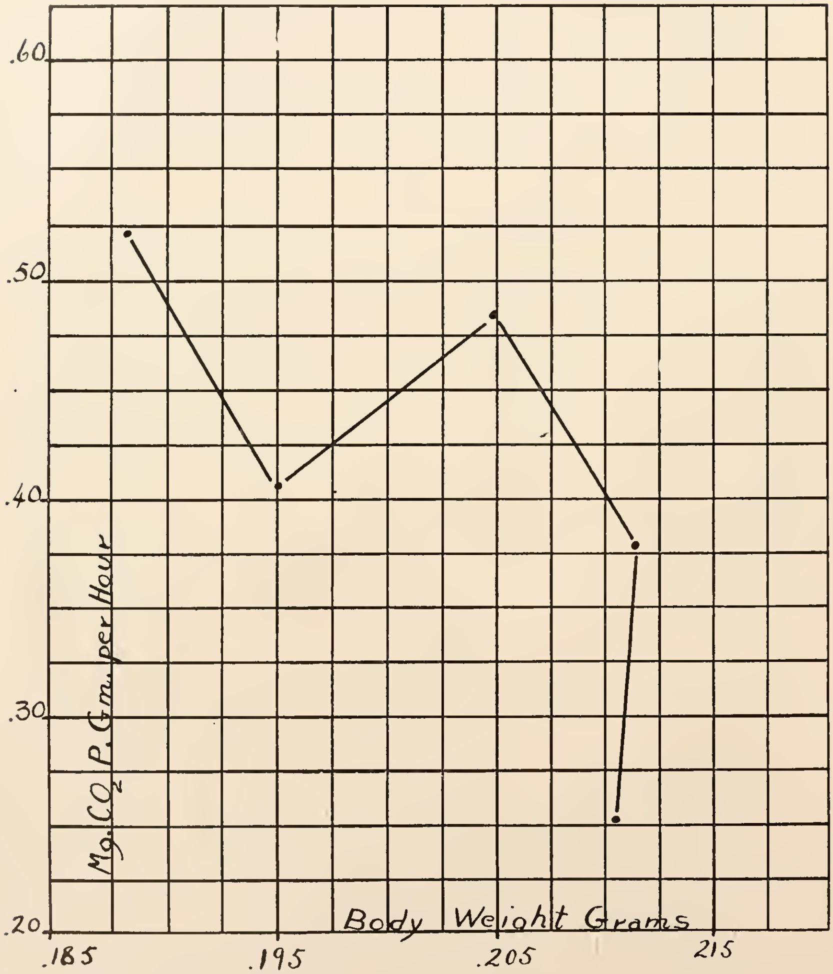
TABLE II.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING STARVATION.

Time interval—7 days.

Average Weight of 1 Insect in Grams.			Average Weight.	Milligrams CO_2 Output per Hour per Gram Body Weight.			Average CO_2.	
Set 1.	Set 2.	Set 3.		Set 1.	Set 2.	Set 3.		
.194	.181	.185	.188	.4252	.6077	.5747	.5338	Normal.
.195	.183	.190		.4512	.5809	.5632		
.195	.197	.192	.195	.3333	.4187	.4988	.4169	Starved 1 week.
.194	.220	.201	.205	.4670	.5200	.5073	.4981	Starved 2 weeks.
.199	.231	.205	.212	.4208	.3238	.4033	.3826	Starved 3 weeks.
.200	.229	.205	.211	.2200	.2882	.2671	.2584	Starved 4 weeks.

set the output increased during the second week. The explanation of this increase is questionable. It may be that at this stage the nymph draws heavily on its reserve food or on its tissues. During the third and fourth weeks, the animals showed a steady decrease in metabolic rate. Nearly all the starved nymphs died before the seventh week, although two lived two months without food.



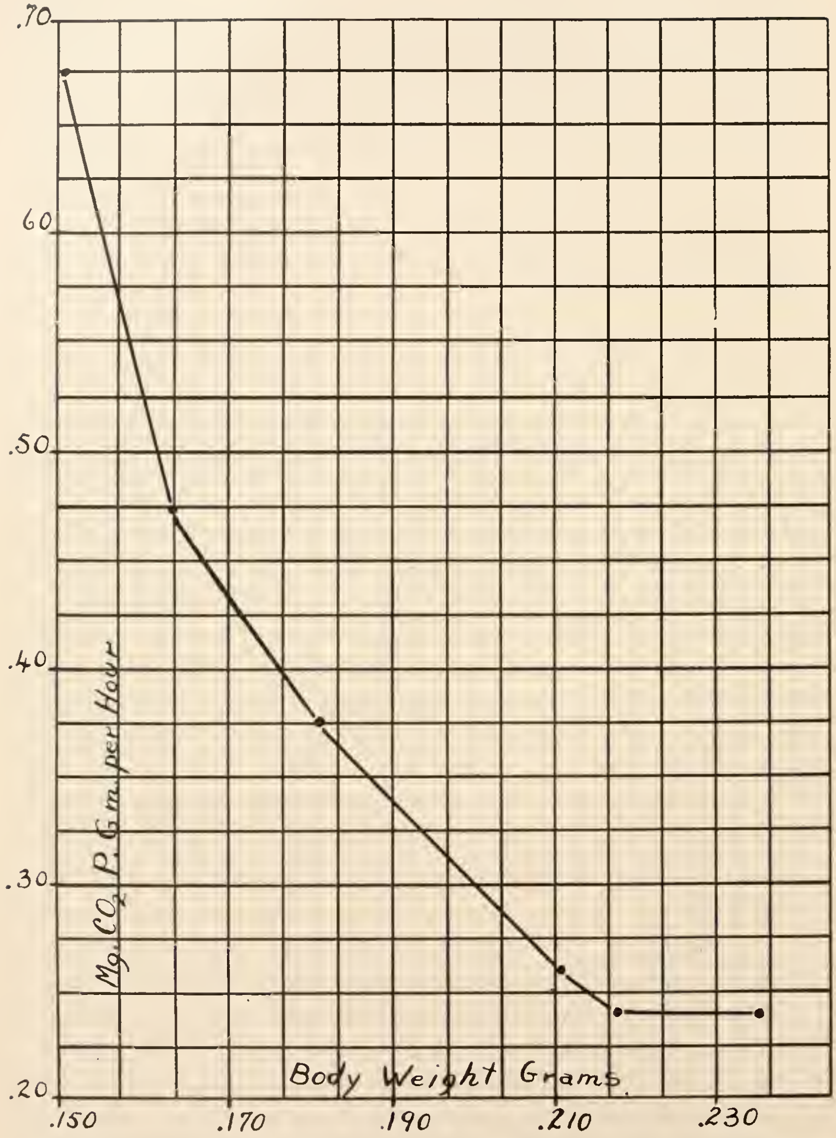
GRAPH 2. Curve shows rate of CO_2 output during four weeks of starvation. Time interval 7 days. Temp. 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

In comparing this graph with the graph of controls (Graph 1), it is noticed that the carbon dioxide output of a starving insect decreases in one week at an amount comparable to the decrease in twenty days in a younger insect and to ten days in the older animals. At the end of four weeks of starvation, the carbon dioxide output is about the same as the output of the controls at the end of three months. It is interesting also to note an increase in weight in the starving insects. Since the nymphs are aquatic, this increase is probably due to the replacement of reserve fat by water during starvation.

C. *The Effect of Darkness on Carbon Dioxide Output.*

The carbon dioxide output of three sets of nymphs was determined under normal conditions of light and then the nymphs were placed in darkness. Darkness was maintained by placing a cap of heavy black paper over the finger bowls. The paper was of the type used to protect photographic paper from the light and was admirably suited for the present purpose. Air could pass under the edge of the bag, which was fastened here and there to the table. The temperature remained around 22° C. and the animals were fed as usual, but in a dark room. Sticks were used as the objects upon which the nymphs could cling. Water plants would decay in the dark and offer a possibility of toxic materials being added to the water. During experimentation the room was in darkness and the experimental jar and the water bath were covered with black paper. Once a week the carbon dioxide output was determined and the results are recorded in Table III. and Graph 3.

The metabolic rate decreased progressively with the weeks of darkness up to the sixth week, when four individuals died. Although the experiment was then brought to a close, the remaining nymphs were kept in the dark until all died, which occurred within a period of two weeks. That the metabolic rate of these animals is decreased by darkness can readily be seen by comparing the data here given with the data obtained from the controls. Per unit of weight the CO_2 output of the nymphs was much less than that of the control animals. A control insect weighing .214 gram gave off .6499 milligram of CO_2 per



GRAPH 3. Curve shows rate of CO_2 output in darkness. Time interval 7 days. Temp. 22° C. Abscissas, gram body weight per insect. Ordinates, milligrams CO_2 per gram insect per hour. For further explanation see description in text.

TABLE III.

SHOWING THE ACTUAL CO_2 OUTPUT IN MILLIGRAMS PER GRAM BODY WEIGHT PER HOUR, DURING DARKNESS.

Time interval—7 days.

Average Weight of 1 Insect in Grams.				Milligrams CO_2 per Hour per Gram Body Weight.				
Set 1.	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.	
.158	.165	.173	.165	.6613	.7650	.5999	.6754	Light.
.164	.170	.181	.172	.4359	.5931	.4071	.4787	1 week in dark.
.182	.181	.193	.185	.3626	.4207	.3404	.3746	2 weeks in dark.
.211	.199	.207	.206	.2345	.3093	.2303	.2580	3 weeks in dark.
.218	.219	.214	.217	.2523	.2641	.2298	.2487	4 weeks in dark.
.233	.225	.229	.229	.2317	.2207	.2177	.2234	5 weeks in dark.

hour, while an insect in darkness weighing .211 gram produced only .2345 milligram.

Since most of the animals lived but six or seven weeks, the decreased CO_2 production must have been accompanied by a pathological condition resulting in early death. It is evident that light is necessary for the normal physiological processes, and that a lack of it produces an abnormal condition bringing about death. It would be interesting in further research to determine what part of the spectrum is of greatest importance in the normal life of these nymphs.

D. Carbon Dioxide Output with Rising Temperature.

Table IV. gives the actual amounts of CO_2 given off by three sets of insects as the temperature of the water in which they were living was gradually raised. The average figures are shown in Graph 4.

Most of the authors already cited determined the CO_2 output at each increase in temperature without making a study of the metabolic rate after a longer exposure to the same temperature. Instead of making the usual successive determinations at different temperatures, three determinations were made at each temperature. These records were made after twenty-four, forty-eight, and seventy-two hours' exposure to the given temperature. In following this plan, a different curve was secured than is usually drawn. Reference to the graph shows that the CO_2 output goes up for each temperature after the twenty-four hours of exposure, but goes down again after the longer periods of exposure. The output is about normal after the seventy-two hours at the given temperature. These results were found to be true in the case of each rise in temperature. If one connected with a line the twenty-four-hour points on this graph, the usual rising curve could be secured. However, according to the method of determination followed here, the insects show a mechanism for adjustment or acclimatization as evidenced by their carbon dioxide output.

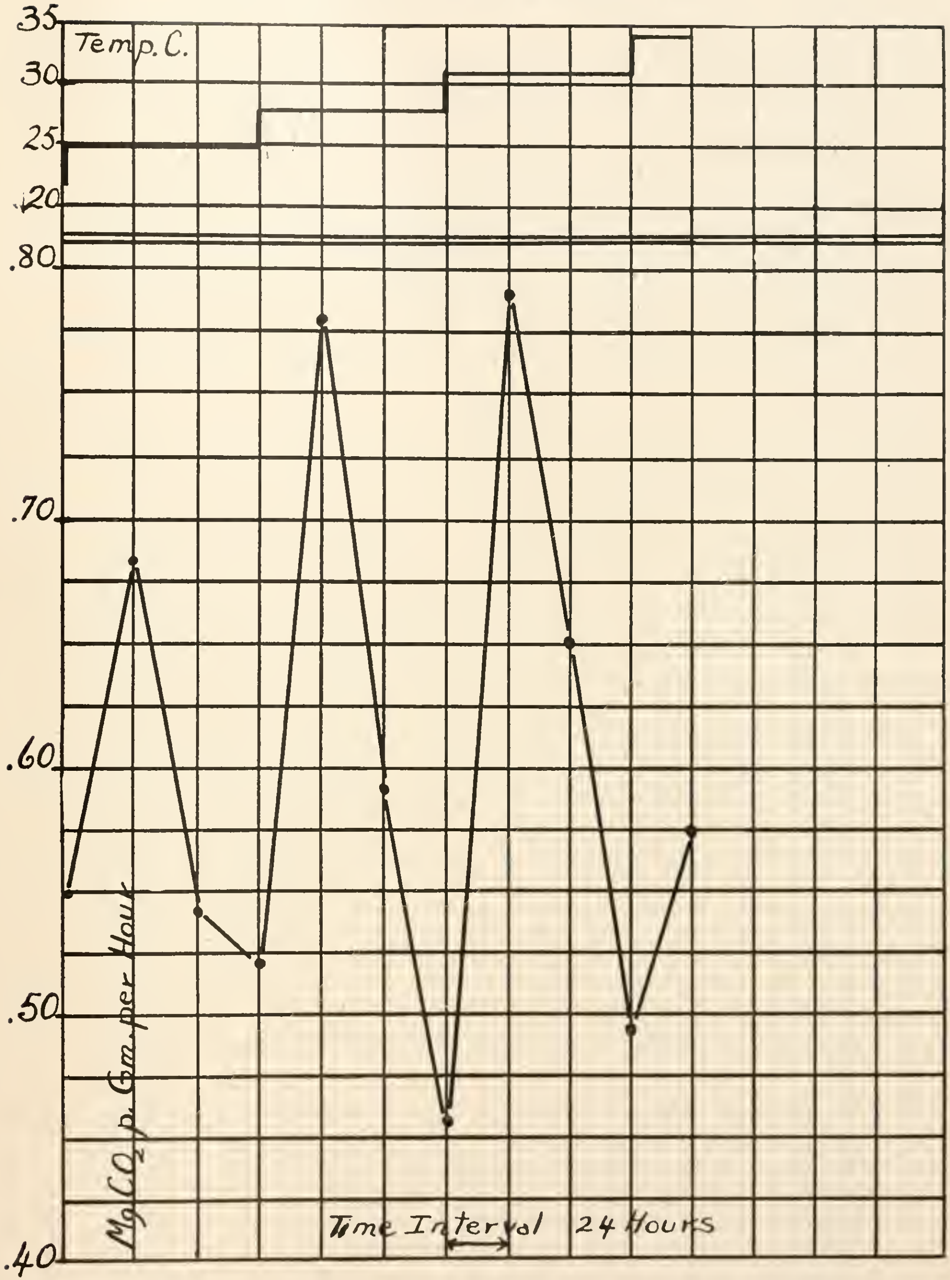
Table IV.

Showing the Actual CO_2 Output in Milligrams per Gram Total Body Weight per Hour, during Periods of High Temperature.

Temp.	Time in Hours.	Weight of 1 Insect in Grams.				Milligrams CO_2 per Gram Body Weight per Hour for 1 Insect.			
		Set 1.	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.
22		.148	.165	.161	.164	.6087	.5579	.6091	.5499
22		.163	.170	.177		.4723	.5341	.5173	
25	24	.163	.170	.177	.170	.6518	.6742	.7155	.6805
25	48	.163	.173	.177	.171	.4538	.5404	.6496	.5483
25	72	.165	.173	.182	.173	.4320	.5511	.5774	.5202
28	24	.171	.174	.182	.176	.7655	.7915	.7910	.7827
28	48	.171	.177	.182	.177	.4875	.6731	.6101	.5902
28	72	.177	.179	.184	.180	.3946	.5070	.4978	.4665
31	24	.178	.181	.184	.181	.7746	.7999	.7899	.7881
31	48	.178	.181	.184	.181	.6637	.6359	.6449	.6482
31	72	.178	.181	.186	.182	.4400	.5252	.5088	.4913
34	24	.175	.183	.186	.181	.4914	.5100	.6666	.5560

The greatest output is between 28° and 31° C., this representing for the nymphs the maximum in catabolic reactions. Above 31° the temperature has a deleterious effect upon the organism, for the insects respond but slightly to a further rise in temperature. At this high temperature, the decreased carbon dioxide production is no doubt accompanied by a physiological injury, for the animals died after twenty-four hours in water at 34°.

After twenty-four hours at thirty-one degrees, the oxygen content of the water was determined. It was found that the water still contained around 5.9 parts per million.



GRAPH 4. Curves show rate of CO_2 output at high temperatures. Abscissas, time in hours. Ordinates, at top, temperature degrees Centigrade; at bottom, milligrams CO_2 per gram body weight per hour. For further explanation see description in text.

E. The Effect of Low Temperature on Carbon Dioxide Output.

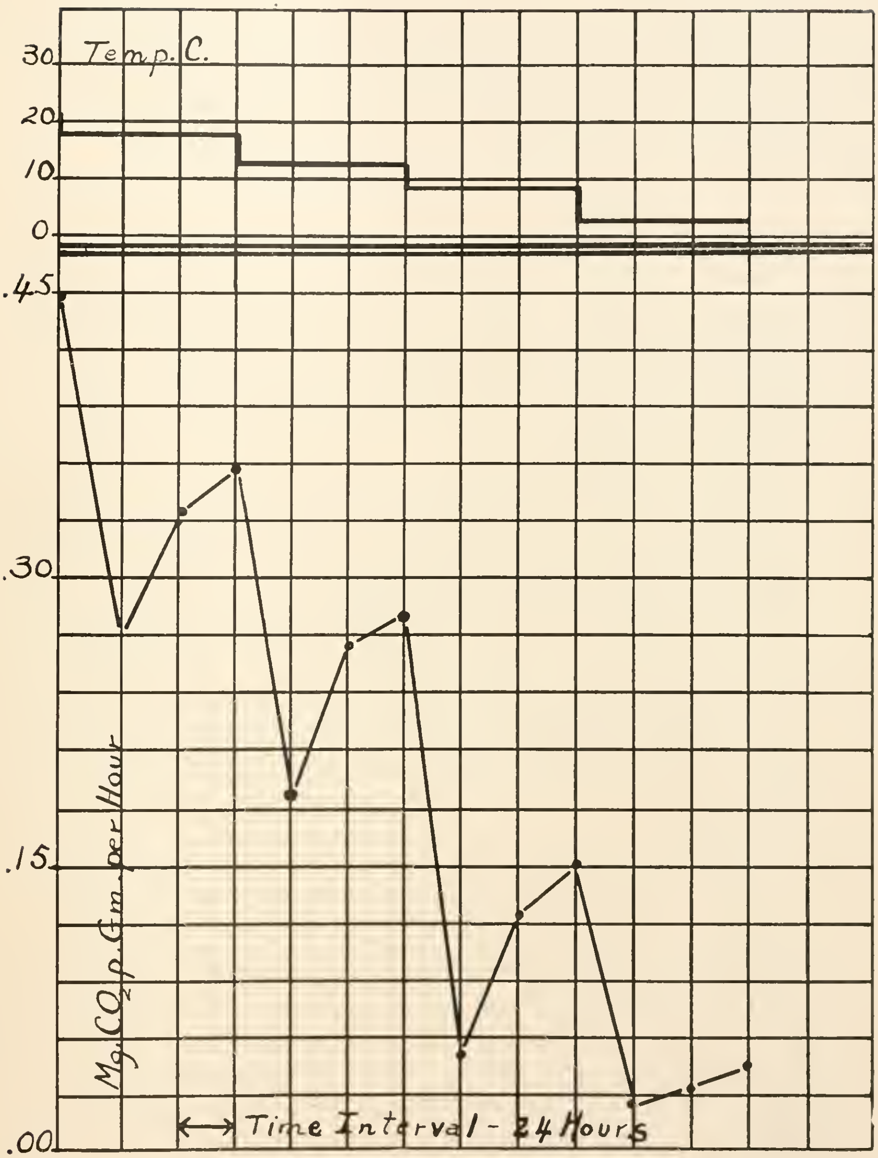
The insects used in the experiments to show the effect of low temperature on CO_2 output were somewhat larger than the insects used in the previous experiments. The former experiments were carried on in the fall months, while the present experiments were carried on in the winter, since it was thought advisable to use running water from the lake at a time when it maintained a constant temperature of 6°. Comparisons may be made, however, with insects of similar weight in the controls, to determine the relative amounts of carbon dioxide given off.

Table V.

Showing the Actual CO_2 Output in Milligrams per Gram Total Body Weight per Hour, during Periods of Low Temperature.

Temp.	Time in Hours.	Weight of 1 Insect in Grams.				Milligrams CO_2 per Gram Body Weight per Hour for 1 Insect.			
		Set. 1	Set 2.	Set 3.	Average.	Set 1.	Set 2.	Set 3.	Average.
22°		.258	.243	.211	.240	.4263	.4750	.5001	.4548
22°		.266	.246	.217		.4135	.4519	.4722	
17°	24	.260	.246	.217	.241	.2538	.2891	.2906	.2778
17°	48	.266	.246	.217	.243	.3501	.3477	.3404	.3460
17°	72	.266	.250	.221	.246	.3721	.3655	.3494	.3623
13°	24	.278	.250	.221	.250	.1978	.1861	.2054	.1964
13°	48	.278	.250	.221	.250	.3079	.2737	.2977	.2931
13°	72	.286	.253	.227	.255	.3165	.2999	.3147	.3104
9°	24	.288	.253	.228	.256	.0593	.0577	.0694	.0621
9°	48	.288	.253	.228	.256	.1337	.1272	.1399	.1336
9°	72	.289	.260	.237	.262	.1582	.1488	.1577	.1549
3°	24	.289	.260	.237	.262	.0380	.0299	.0376	.0352
3°	48	.289	.260	.237	.262	.0425	.0471	.0517	.0471
3°	72	.289	.264	.237	.264	.0570	.0441	.0597	.0536

Table V. shows the carbon dioxide output of the nymphs as the water temperature was lowered gradually from 22° to 3° C. The same scheme of duration of time at each temperature was used as previously employed in the records with rising temperature. Graph 5 shows that the carbon dioxide output decreased progressively with falling temperature after twenty-four-hour durations, but also shows an acclimatization to a low temperature after being exposed to it for a longer period of time. The carbon dioxide output drops for each drop in temperature, but the output increases again in forty-eight and seventy-two hours at a



GRAPH 5. Curves show rate of CO_2 output at low temperatures. Abscissas, time in hours. Ordinates, at top, temperature degrees Centigrade; at bottom, milligrams CO_2 per gram body weight per hour. For further explanation see description in text.

given temperature. At 3°, the metabolic processes are going on at a very slow rate and acclimatization at this temperature was only slight. Low temperature did not affect the nymphs in any permanent way, for they lived a normal length of time after the experiment was completed.

Summary and Conclusions.

Although a great deal of work has been done on the metabolism of insects, very little has been published on the metabolic rate in truly aquatic forms. As far as I know, nothing has appeared relative to the carbon dioxide output of Æshnid nymphs. Their respiratory mechanism is an interesting one and the present work is only an introduction to the many related research problems that present themselves.

1. During the nymphal stage of *Æshna umbrosa*, the carbon dioxide output decreases progressively as the nymphs grow older. The younger, more active individuals have the higher rate of respiratory output per gram body weight.
2. The CO_2 output of starved animals decreases during the period of starvation, except during the second week, when the metabolic rate is higher than the normal.
3. Darkness has a marked effect on the metabolism of these animals. The CO_2 output decreases steadily with the weeks in darkness and the physiological condition is so affected, that the nymphs die.
4. Higher temperatures cause increased rates of CO_2 output and lower temperatures tend to have the reverse effect. However, after an initial increase in CO_2 output following twenty-four hours of exposure to the high temperature, the output decreases again after longer exposure to the same temperature. The same effect is evidenced by the nymphs exposed to low temperature, *i.e.*, decrease in CO_2 output is followed by an increase after a longer period at the same temperature. The insects indicate a mechanism for acclimatization.

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FURTHER OBSERVATIONS AND EXPERIMENTS ON THE SYMBIOSIS BETWEEN TERMITES AND THEIR INTESTINAL PROTOZOA.

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It has been possible through a grant from the Bache Fund of the National Academy of Sciences and the collecting ability of Dr. Harold Kirby, Jr., to extend somewhat my observations on the symbiosis between termites and their intestinal protozoa.

Hitherto the investigations (Cleveland, '24, '25*a*, '25*b*) have been confined principally to two genera, *Termopsis* and *Reticulitermes;* similar observations have now been made on six more genera (subgenera according to some investigators), with fragmentary observations on several other genera. All the termites in this investigation except *Reticulitermes* came from Panama and Costa Rica and were identified by Dr. T. E. Snyder.

In most of the experiments the protozoa were removed by confining the termites in oxygen at a pressure of 60 pounds for one hour; in a few instances higher pressures for a shorter period and lower pressures for a longer period were used. It was never possible, regardless of how high a pressure was used, to remove all the protozoa in less than ten minutes. The termites, as in previous experiments (Cleveland, '25*a*, '25*b*), showed no ill effects from the oxygen even if confined in it for a much longer period than that required to kill all their intestinal protozoa.

Large numbers of termites were used when available and after the removal of the protozoa they were kept in large Petri plates and were fed either cellulose (filter paper) or wood. Moisture was supplied in all except the earlier experiments by placing in each Petri plate three small pieces of absorbent cotton dipped in water and then mashed between the fingers until as much water as could be gotten out in this way was removed. Every third day the pieces of cotton were remoistened. This appears to be the best way to keep termites in the laboratory so that they may be constantly observed with little effort and without being greatly disturbed. When fed wood, they were not given enough to hide themselves by burrowing into it. They prefer the wood and paper and do not eat the cotton unless the supply of wood or paper is exhausted. Death due to molds—the bane of poorly kept laboratory termites—may be avoided entirely when termites are kept in this manner. Controls of all the experiments were kept in the same manner and are in excellent condition now, ten months after the defaunated termites were all dead.

The principal results lend themselves rather easily to tabulation and are set forth in Table I. These data are not as complete as they should be. They show, however, that none of these termites are able to live indefinitely after their protozoa have been removed by oxygen treatment. But, in most cases, they do not show why some termites lived longer than others. This may be a difference inherent in the termites themselves, some being more dependent on protozoa than others. Further investigations, in fact, may even show that certain protozoa-harboring termites are able to live for a long time, if not indefinitely, on a normal diet of wood after their protozoa have been removed. The reason why the termites of some genera lived so much longer than those of other genera may also, in part, be a matter of food. Too few termites were available in some instances to properly control this factor. Some of the termites were strikingly different in appearance and behavior from others, and we may well expect considerable differences in their ability to maintain themselves on the same diet after the removal of their protozoa, but it will be necessary to carry out more extensive experiments before this can be definitely demonstrated. However, a large number of experiments were carried out with *Reticulitermes* under ideal conditions with the result that this termite of the family Rhinotermitidæ was not able, on the average, to live nearly so long as termites of three genera of the family Kalotermitidæ (*Neotermes, Kalotermes, Cryptotermes*). In the case of *Reticulitermes*, the reason why some of the experiments ran so much longer than others is quite clear. Those experiments which ran so long

TABLE I.

SHOWING APPROXIMATE LENGTH OF LIFE IN DAYS OF VARIOUS DEFAUNATED TERMITES.

Termites.	Number of Experiments Carried Out.	Number of Termites in Each Experiment.	Days before All Termites in One Experiment Were Dead.	Days before All Termites in All Experiments Were Dead.	Average Duration of Experiment or Length of Life of Defaunated Termites.	Food.	Remarks.
Reticulitermes flavipes Kollar	20	200	18	68	29	Paper, sound wood, decayed wood.	Kept in Petri plates. Those fed decayed wood lived longest.
Calcaritermes brevicollis Banks	5	50	19	29	26	Sound wood.	Kept in jars.
Cryptotermes dudleyi Banks	3	50	52	77	66	Sound wood.	Kept in Petri plates.
Lobitermes longicollis Banks	2	200	20	22	21	Sound wood.	Kept in jars.
Neotermes holmgreni Banks	4	50	94	123	107	Paper.	Kept in Petri plates.
Glyptotermes barbouri Snyder	3	50	26	28	27	Sound wood.	Kept in large test tubes.
Kalotermes panamæ Snyder	2	300	71	80	75	Fairly well-decayed wood.	Kept in Petri plates.
Kalotermes tabogæ Snyder	2	50	129	165	147	Paper	Kept in Petri plates. Only a few lived so long.

contained a fairly large number of second form young adults. These individuals are fed and supported by the workers and nymphs (Cleveland, '25*b*). They had few, if any, protozoa to lose (Cleveland, '25*b*) and were able to exist in a fairly normal manner on the salivary secretions of the workers and nymphs for three weeks or more, or as long as the workers and nymphs lived and were able to feed them, and then came their period of starvation followed by death three to four weeks after the workers and nymphs who supported them died.

The conditions under which *Lobitermes*, *Calcaritermes*, and *Glyptotermes* were kept were perhaps not so favorable as the conditions of the other genera of this family (Kalotermitidæ), *Kalotermes*, *Cryptotermes*, and *Neotermes*, and perhaps for this reason they did not live so long. These experiments were carried out before the Petri plate method of keeping termites was discovered, and all termites of these genera were used up.

Some material of two other genera, *Leucotermes* and *Coptotermes*, of the family Rhinotermitidae was procured, but these termites behaved so differently from *Reticulitermes* and the genera of the Kalotermitidæ that it was impossible to carry out the same experiments with them. One or two days in closed jars or test tubes usually resulted in their death. Almost any kind of handling, especially confinement in a closed jar or, sometimes, shipment for eight to ten days in a wooden box, resulted in the loss of their intestinal protozoa. Owing to the fact that material of these genera was not plentiful, no attempt to study them in aërated containers was made. It will be very interesting indeed for comparative purposes, and possibly for gaining some definite information on the origin of termite symbiosis and parasitism, to carry out a large number of experiments on these and other genera of the Rhinotermitidæ. Valuable information in this same direction may also be obtained by a more critical study of the ability of several genera of the Kalotermitidæ to live on various diets after the removal of their protozoa. It is also very important to study the single genus *Mastotermes* of the most primitive family, the Mastotermitidæ.

Several genera of the most highly specialized family, the Termitidæ, were obtained. This family as a rule (Cleveland, '23*a*, '26) does not harbor protozoa, although a few small flagellates and fairly large xylophagous amœbæ[1] occur in some species, but perhaps not in sufficient numbers, for the most part, to be of any decided advantage to their hosts. Many species in this family do not feed on wood at all, and those that do, feed on more decayed wood than most termites of the other three families. Numerous attempts have been made to feed cellulose (filter paper) to several genera of the Termitidæ (*Anoplotermes*, *Mirotermes*, *Microcerotermes*, *Cornitermes*, *Orthognathotermes*, *Armitermes*, *Convexitermes*), but with no very definite results. These termites eat moistened filter paper readily and it will be an easy matter to determine whether or not they can digest it as soon as a convenient method for keeping them in the laboratory is worked out. In most of the experiments that were carried out the controls, or those termites fed very much decayed wood and fresh soil or humus, lived but little longer than those fed paper. When the termites were fed paper and allowed to remain in the nest or a portion of it, they lived much longer than when placed in jars with paper only. This was perhaps due to the fact that the fungi in the nest aided them in some way in the digestion of food. However, the laboratory conditions at best were too different from those of nature to lend much value to the experiments. When better means of keeping the Termitidæ in the laboratory are obtained, this subject will be considered again.

Termites of all four families harbor very many spirochætes, which, like the protozoa, may play a rôle in the digestion of cellulose and hemicellulose. Considerable time has been given to an attempt to remove the protozoa from several genera of the Kalotermitidæ without removing the spirochætes, but with no very definite results, because practically every treatment that removes the protozoa removes the spirochætes also. It has been possible, however, in a few experiments to remove the protozoa by heat treatment without killing the spirochætes, but they were all dead three or four days later. It will be necessary to repeat these experiments many times under easily controlled conditions before any definite conclusions are drawn regarding the relation of the spirochætes to the termites and to the protozoa. They may live in some sort of a symbiotic relation to the protozoa—we do not know. They are attached to the protozoa—a fact overlooked by Damon ('26) and by Hollande ('22), who have recently given brief descriptions of termite spirochætes. Very few of the spirochætes, indeed, except those of the large form similar to the *Cristispira* of oysters, are free in the intestinal fluid. Countless millions are often attached to a single protozoön, which seems scarcely able to move. Some protozoa have no attached spirochætes, while others of the same species may have from one individual to several millions attached to them. Sometimes half of the protozoa in a termite are completely covered with spirochætes, which, of course, may be mistaken for flagella unless examined by the dark field method. Spirochætes may also at times be seen inside the protozoa when the termites have been fed paper for a long period. All attempts to grow the spirochætes have failed so far, and all animal inoculations have been negative. The subject is being pursued further and will be reported on in more detail later.

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[1] These amœbæ will be described by Dr. Harold Kirby, Jr.

Somewhere in the 1,500 species of termites there is a key to the unique and most interesting animal association throughout all nature. There have probably been many gradatory steps in the formation of the association, but perhaps enough of the steps remain today to enable us, after many years of investigation, to trace the order of events. The fact that in the Termitidæ we find a few protozoa in a species here and there suggests that in this family we are now viewing either the beginning or the end of a widespread symbiotic association. The ease with which those genera of the Rhinotermitidæ resembling the Termitidæ closely in behavior lose their protozoa when handled in the laboratory or when packed and shipped, indicates that possibly the Termitidæ are losing their protozoa.

ADDENDUM.

Since this paper went to press termites have been fed cellulose thoroughly moistened with a five per cent. aqueous solution of acid fuchsin and it has been possible in this way to remove all spirochætes from them without doing any damage whatever to the protozoa or to the termites. Some of the termites have been spirochæteless now for three months and it is impossible to detect any deviation from their normal appearance and activity, even though they have been fed continuously on the diet which removed their spirochætes. Also no change has occurred in the number and activity of the protozoa. Evidently the spirochætes play little, if any, role in the digestion of wood and cellulose. These results will be reported in detail later.

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NOTES ON A 32 MILLIMETER FREEMARTIN.

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In an earlier paper, Bissonnette ('24) described the development of the reproductive ducts and canals in cattle embryos from 28.0 mm. crown-rump length to 20.0 cm., and compared with that series one of 5 freemartins from 9.0 cm. to 20.0 cm. In that paper the gonad of a 9.0 cm. freemartin was described which showed unmistakable development of ovarian cortex or secondary sexcord region. The material described gave support to the theory of Lillie ('16 and '17) and Keller and Tandler ('16) that the freemartin is a female modified in utero in the male direction by the action of a male hormone from the testis of her twin brother. It showed that she may go so far in the female direction as to have a secondary sexcord region and even to the condition with an intermittent wolffian duct before undergoing regression of the müllerian duct derivatives and added growth and differentiation of the so-called "male" structures. The one there described was the first showing such sexcords. Since earlier writers had agreed that such cords were never found in freemartins (Chapin, '17, Willier, '21, Lillie, '23) the question remained as to whether this was not a very abnormal situation among freemartins. Further study of younger stages of the freemartin was needed to answer this question.

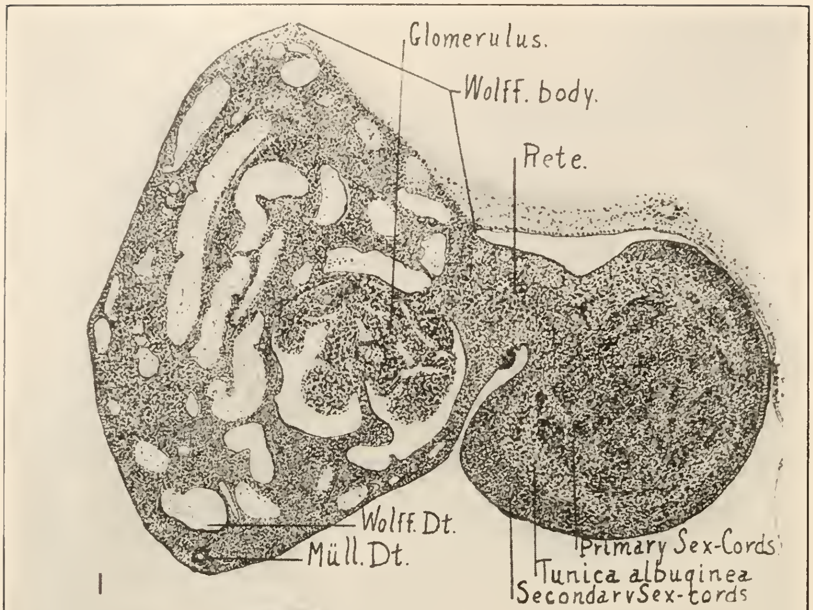
Lillie ('23) described and figured a 3.75 cm. freemartin with no such cords but with a germinal epithelium more than one cell thick—in some places even 3 or 4 cells thick—but never more than about a third to a sixth of the thickness of the corresponding layer in the single-born female. He found the ducts and canals in no way different from those of the female of corresponding size. This was the smallest stage of the freemartin described and it showed definite modification of the gonad in the male direction.

The subject of the study described in this paper is a freemartin of 32 mm. crown-rump length which is of interest because (1) it is the smallest stage so far studied, (2) it has a minimum of union of the chorions compatible with any blood transfusion, (3) it is another case where ovarian cortex is found outside the tunica albuginea, (4) it appears to be as near as possible to the stage of distinguishable sex-differentiation in which modifications of the freemartin type can be distinguished, (5) it shows slight freemartin modification and forms the initial end of a series of intergrades between the postnatal freemartin types and the normal female.

Material and Methods.

This 32 mm. freemartin, twin to a 34 mm. male, was taken July 28, 1923, at the killing floor of Swift & Co., Chicago Stockyards. Since it was the smallest freemartin in over 50 taken up to that time by the writer, it was especially carefully treated. The uterus of the mother was slit open throughout both horns and the membranes and twins carefully lifted out. The embryos were far up the opposite horns of the uterus and the chorions were but slightly united and showed no bloodvessel union visible to the naked eye. The placentæ pulled apart with a small amount of bleeding only, when they were handled after removal, though they held together during that operation. Chorions and embryos entire were placed at once into Bouin's fluid which was changed once about two hours after they were put in. No injection was attempted since the vessels were judged too small for the apparatus at hand on the killing floor and the help available, and the internal organs were wanted for microscopic work and early fixation was desired. The membranes and embryos were washed in 50 and 70 per cent. alcohols, to the latter of which at its second addition a few drops of a saturated aqueous solution of lithium carbonate was added to help remove the picric acid. They were then stored in 80 per cent. alcohol for about a year and a half, when the embryos were removed and the posterior parts of both embryos were stained in bulk in alum cochineal and sectioned 30 micra thick. The series was counterstained on the slide in orange G and mounted in balsam.

Both maternal ovaries had been taken by the boy who collects those with large corpora lutea for extraction, so it is supposed each had a corpus luteum.


Glomerulus.
Wolff. body.
Rete.
Wolff. Dt.
Müll. Dt.
Primary Sex-Cords
Tunica albuginea
Secondary Sex-cords
1

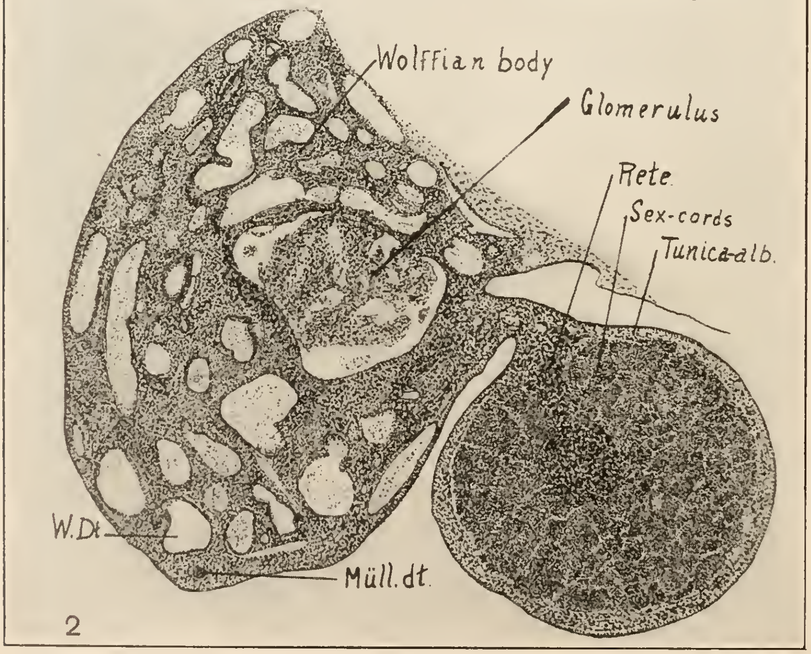
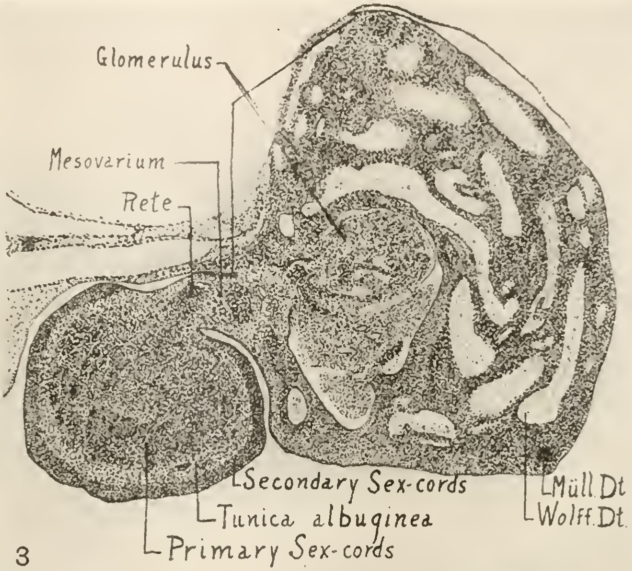

Wolffian body
Glomerulus
Rete.
Sex-cords
Tunica-alb.
W. Dt
Müll. dt.
2

FIG. 1. Transverse section through gonad, mesonephros, and ducts of right side of normal female, 32 mm. crown-rump length. × 38.3.

FIG. 2. Transverse section through right gonad, mesonephros, and ducts of normal male, 32 mm. crown-rump length. × 38.3.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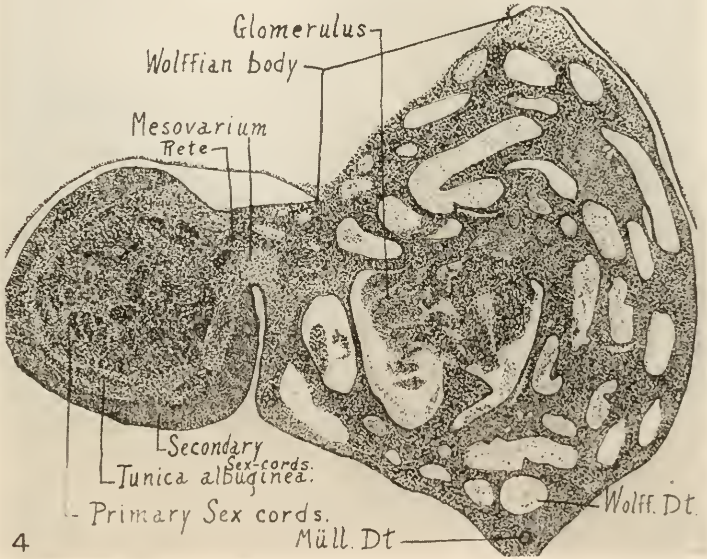
Mesovarium
Rete
Secondary Sex-cords
Tunica albuginea
Primary Sex-cords
Müll. Dt
Wolff. Dt.

3


Glomerulus
Wolffian body
Mesovarium
Rete
Secondary Sex-cords.
Tunica albuginea.
Primary Sex cords.
Wolff. Dt.
Müll. Dt

4

FIG. 3. Transverse section through left gonad, mesonephros, and ducts of freemartin, 32 mm. crown-rump length. × 34.3.

FIG. 4. Transverse section through left gonad, mesonephros, and ducts of normal female, 32 mm. crown-rump length. × 34.3.

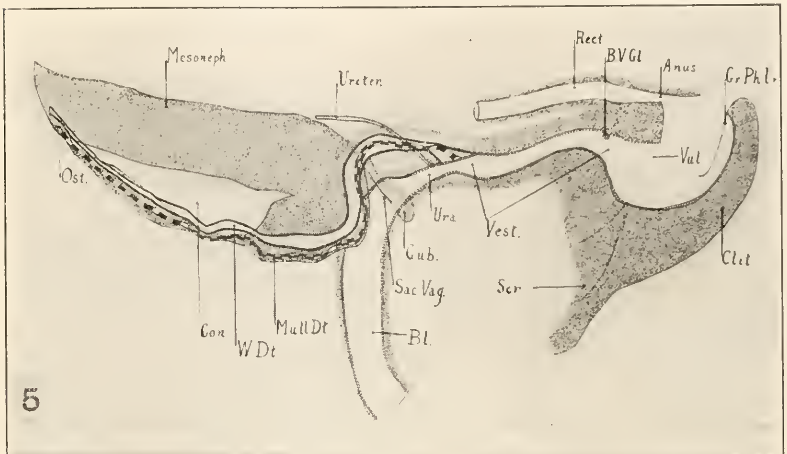
On washing out, no vascular anastomosis could be made out with the binocular dissecting microscope, low power, but the bleeding on separation was evidence that there was capillary connection at least.

Previous work had shown that thick sections have some advantages over thin in showing the zones in the gonads and regions of even slight degeneration with consequent pigment production. They also save time in reconstruction of the ducts and canals. Such reconstruction was made for this freemartin and for a normal female of the same size for a control, after the manner of Bissonnette ('24). The notochord was used as reference line for dorso-ventral relations of ducts, canals, mesonephros, and anlagen of the round ligament or gubernaculum, and gonads (Figs. 5 and 6). Typical cross-sections through the gonad, mesonephros, müllerian and wolffian ducts, in the normal female, male, and freemartin, are shown in Figs. 1, 2, 3, and 4.

Description and Comparison with Normals.

The Placental Situation.—The twins were far apart in discrete but united chorions with cotyledons beginning to appear as opaque patches. One chorion was telescoped slightly into the end of the other and they were so united that bleeding occurred on separation. Otherwise the chorions were normal. Both older and younger cases have been taken by the writer in which the chorions were in contact but not so adherent as to cause bleeding on separation. In all such cases so far investigated the female has proved normal. This situation was much the same as that in the 9.0 cm. freemartin No. 1, Fig. 2, of Bissonnette's ('24) paper, where a slight though definite amount of modification in the male direction was demonstrated and secondary sexcord regions or cords of Pflüger were found. It is another case permitting a small blood interchange between the twins and a relatively small concentration of hormone would be expected on the female side and so a longer period might elapse before the effective minimum concentration of the hormone is reached. The amnions were separated widely and were normal.

The External Genitalia.—Externally this freemartin was female in type as shown by Figs. 5 and 6 in sagittal section. The clitoris was slightly more recurved than that of the normal control. However there are variations in this respect among single females due to the difference in relative curvatures of the embryos at the time of killing and fixing. It resembles the one figured by Lillie ('23) for his 3.75 cm. one (Fig. 5) externally as well as in gross internal dissection. So it is not figured here in these aspects. The urogenital aperture is open although in the male of the same size and in the male twin it is closed and has a raphe


Mesoneph
Ureter
Rect
BVGl
Anus
Gr Ph l.
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Ost.
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Vest.
Gub.
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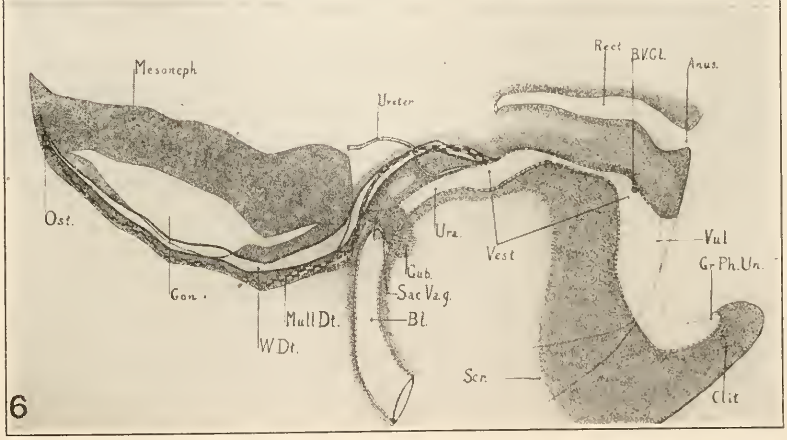
Sac Vag.
Scr
Con
Mull Dt
W Dt
Bl.
5


Mesoneph
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Vul
Gr Ph.Ur.
Gub.
Sac Vag.
Bl.
Con.
Mull Dt.
W Dt.
Scr.
Clit
6

FIG. 5. Reconstruction of the urinogenital system of the freemartin of 32 mm. crown-rump length. × 9.5.

FIG. 6. Reconstruction of the urinogenital system of the normal female of 32 mm. crown-rump length. × 9.5.

to mark the midline in place of a slit. The perineum is not expanded ventrally as in the male.

The vulva, vestibule, and bulbo-vestibular glands are female in type and proportions (Figs. 5 and 6). The ureters also come off the urethra or urogenital sinus as in the female from a ridge in the dorsal wall.

The wolffian ducts are not much affected though their lumen is greater just posterior to the mesonephros than in the control. It is not to be expected that they would be affected greatly since they are still the ducts of the functional mesonephros.

The müllerian ducts are not so large in section as the control and lack a lumen in some regions where there is one in the control. They appear to be intermediate between the male and the female and, as in the male, show some signs of regression. Their staining reaction is like that of the male (Figs. 1, 2, 3, 4). This difference in size is most apparent in the region just posterior to the mesonephros where the wolffian ducts are larger. The slight difference in the type of entry into the vestibule shown in Figs. 5 and 6 is probably not significant as such differences are found in single females. The ostia tubæ abdominale at the anterior end of the müllerian ducts open out flat with lips on the ventral aspect of the anterior end of the mesonephros and show a tendency to be associated with the rete there. This rete appears to lead over into the mesonephros and to join a glomerulus as in the normals of both sexes at this age.

The Gonads.—Figs. 1 and 2 are from sections through near the middle of the right gonads of 32 mm. female and male respectively, and figures 5 and 6 are similar sections of left gonads, etc., of the same sized freemartin and female, drawn with camera lucida to the same scale. As stated above, these thick sections (25 and 30 micra) show up the regions of the gonad better than thin sections, and cortical regions stand out better. The gonads of the freemartin have the same relations to the mesonephros as those of the female. Rete ovarii are similar in that they do not extend into the middle of the gonad as they do in the male, but are found only near the mesenterial border of the gland (Willier, '21; Bissonnette, '24). The type of tunica albuginea is female but the medulla of the ovary does not show signs of as great activity as in the control. A cortex is present outside the tunica albuginea and is continuous with the germinal epithelium or serosa coat from which it appears to develop, though organization into definite sexcords is not evident in the preparations of either female or freemartin. The secondary sexcord region is not quite so thick as in the control nor is the gonad quite so large in cross-section. It is about 21/23 of the normal in diameter. The length of the freemartin gonad is 1,740 micra while that of the control is 2,150. This may or may not be within the limits of normal variation at this age for no series of measurements of normal ovaries has been made at this stage to determine these limits. The difference in size is apparent from the sections drawn to the same scale. This would be even greater were both sections taken from exactly the same relative level of the gonad. The freemartin was cut 62/100 of the length from the front end and the female 58/100 of the corresponding distance. As shown in the reconstructions, the gonads are larger in diameter posterior to their middle regions but near them. The blood supply of the freemartin and control gonads is distributed through the tunica albuginea inside the cortex, while, in the corresponding male, since there is no cortex outside the tunica, the blood supply is superficial in the definitive tunica albuginea, present at this stage. In the testis at this stage there is marked cellular growth in the medulla or primary sexcord region; in the ovary this activity is less marked; and even less so in the freemartin gonad.

The anlagen of the gubernacula or round ligaments are similar to those of both sexes at this stage. No sex differences in this organ appear till later stages as described by Bissonnette ('24).

DISCUSSION.

Lillie ('16 and '17) and Keller and Tandler ('16) came to the conclusion that the freemartin is a modified female, that sex-determination as a female was consummated at fertilization, and that the abnormalities are due to the action of a male hormone passed to her through the vascular anastomosis in the united placental circulations. They believed that this was a case of normal zygotic sex-determination followed by abnormal sex-differentiation in the female twin. This material supports such a theory by showing a case near the initiation of the differentiation process where the embryo is essentially female in type and condition of the organs, but is slightly underdeveloped in respect to the secondary sexcord region and müllerian ducts. This case, taken in connection with other described cases, particularly where the placentæ were in contact but not united by either capillaries or arteries or veins, as described by Lillie ('17) and Keller and Tandler ('16) and verified by the writer, appears to show that a blood-carried agent or hormone is at work here and not some agent like the "organizer" which Spemann ('25) shows so unquestionably is operative in the early development of the amphibian larva. If such an agent were operating here, mere tissue cohesion or connection would be sufficient to condition full effect, and the great differences between freemartins of the same embryonic age would be difficult to account for except on the basis of variations in zygotic sex-balance of the two individuals of the twins or differences in time of contact of the chorions. Now, in cattle, mere contact is usually attained early by reason of the great elongation of the trophoblastic vesicle; but vascular connection is not always so early, and the effect appears to depend on blood transfusion rather than on tissue continuity.

However, this case and others such as the one described by Keller and Tandler ('16), in which there was slight vascular interchange accompanied by a scarlike welt across the place of junction of the chorions, reducing what they believed was once a greater vascular connection to proportions too small to account for the degree of modification, appear to show that even a capillary anastomosis or an anastomosis of very small bloodvessels is sufficient to condition the modification. The state of the organs appears to depend on the time elapsed since the effective threshold of hormone action is reached, rather than on a varying amount of effective hormone above such threshold. This freemartin and the 9.0 cm. one of Bissonnette ('24) both showed a minimum vascular connection accompanied by a type of modification, or degree of it, which suggests a marked development as a normal female, probably for some time before the hormone became active, followed by regression of the peculiarly female organs and a delayed acceleration of the growth of the essentially male parts. They do not support the assumption that there has been a slower than normal development of the female structures with a slightly faster development of the male ones over the whole period of union, as will appear from what follows.

Lillie's Fig. 11, page 68, of a gonad section from a 3.75 cm. freemartin, in which he found demonstrable vessels connecting the circulations of the twins with no clearly distinguishable place of chorionic union, shows a germinal epithelium together with three or even four layers of cells outside the tunica albuginea in some regions. These cells of the extra layers are irregularly arranged and not in definite cords. These extra cell layers over and above the single layer of cœlomic epithelium homologous to that in the bull of similar size (Fig. 12) he does not interpret as a sexcord region and cords are not possible with so few cells in any case. After inspection of the 32 mm. freemartin in this study and all the freemartins younger than the 9.0 cm. stages examined by the writer microscopically, all of which show the secondary sexcord region, it appears that these extra cells in Lillie's specimen may well be secondary sexcord primordia in which cords have not yet organized. Brambell ('27) finds that in the mouse cords do not organize till later and that in males the germinal epithelium becomes several cell-layers thick after the tunica albuginea forms but later thins out to a single layer. This latter situation does not develop in males in cattle as seen in Fig. 2 of this paper and Fig. 12 of Lillie ('23). Chapin ('17) in some of her gonad sections shows regions which she does not interpret as derivatives from secondary sexcord anlagen, but which appear in the light of this more recent younger material and from an inspection of her slides themselves (kindly loaned by Professor F. R. Lillie) to be derived from such source with possible later proliferation. In any case the secondary sexcord region is not a layer of uniform thickness all over the gonad but is thicker in lateral regions than in ventral. The layer also appears to be resorbed at different rates in different regions and so to remain in some regions longer than in others and possibly permanently. This appears to be correlated with or dependent on pressure by adjacent organs in some way. It is possible for the tunica albuginea to reach the outside of the gonad in some regions and not in others, leaving bands of secondary sexcord derivatives extending lengthwise in the gonad parallel to the medullary cord derivatives. This situation is figured by other workers. These remnants were difficult or impossible to interpret without comparison with the earlier stages or with stages which had undoubted secondary sexcords. Such a series was not available for study by other students of the freemartin. This will be discussed more fully in a later paper on the basis of more material.

From a study of the above mentioned freemartins and of a 6.75 cm. and 7.5 cm. pair from a set of triplets with one bull only (to be discussed in a later paper) where cords of Pflüger are also present but show signs of regression, one is led to conclude that the rate of resorption of these cords is comparatively slow as well as that they may continue to grow in some cases for a short time before regressing. This also leads one to believe that secondary sexcord regions were never present in Lillie's specimen mentioned above, in any greater amount than is shown in his figure (Lillie, '23, Fig. 11, p. 68). This also suggests that the initiation of the male hormone action as inhibitor of the secondary sexcord region was longer delayed in this 32 mm. one than in Lillie's 3.75 cm. one. This may be explained on the basis of the small amount of hormone passing to the female at first through the small vascular connection. This would delay the effective minimum concentration of the hormone. That there is such an effective minimum, in some mammals at least, is shown by the partial castration and grafting experiments of Moore ('21), Sand ('19), Steinach ('20), and many others for postnatal stages. No prenatal testis grafts have yet been possible in mammals so far as the writer can learn.

It is possible that the vascular anastomosis was just becoming established in these twins and that, as the pregnancy progressed, it would have become more effective by the pressing together of the chorions. This would insure a continuous larger supply of hormone on the female side and the rate of modification in this case would be as fast as in those with earlier complete anastomosis, though the condition reached at any time would lag behind that of the others and this freemartin would fall in the group showing small degree of modification as classified by Willier ('21).

Willier ('21) classifies freemartins into three classes on the basis of degree of modification—those with small, medium, and large amount of abnormality—and Lillie states that the freemartins "form an exceedingly well-defined group without intergrades to normal females or normal males." This may be true for postnatal freemartins; but, from the series discussed in this and other papers cited, it appears that a series of intergrades connects the female with the freemartin group, or that the freemartin group moves steadily away from the female group of types, as we pass from younger to older stages. No such series is seen between the freemartin and male. The rare cases of modification externally in the male direction are probably due to upset of some kind in genetic or zygotic sex balance and not to the freemartin type of hormonic modification alone. Such cases occur without twinning in cattle, and in as great a percentage of cases as in freemartins. It is this series in the younger stages leading back to the normal female type among freemartins that so conclusively shows that the freemartin is a modified female and not a modified male. Taken with Lillie's ('17) statistics as to sex-ratios in cattle twins, it furnishes most conclusive proof of the theory of the freemartin of Lillie and of Keller and Tandler.

Lillie ('23) suggests that probably there are individual differences in zygotic balance of sex factors in cattle even in single born calves, and those who have been much associated with cattle breeding and judging, and have seen the differences in potency of males and females inter se, and their differing degrees of male and female "conformation," will agree. This appears to be a fundamental factor in the condition of all freemartins and doubtless works from both sides, male as well as female, in the pair. The hormone, even, may not be equally potent in all cases. Its appearance may be delayed in some males for this reason. So the initial action may be delayed even in cases with early complete intercommunication of blood vessels. The females also may differ in their resistance to the masculinizing action of the male hormone: they may differ already in femaleness. Comparison between this case and Lillie's and other small freemartins illustrates how these factors may lead to variations, among the early stages at least. It is also just possible that the differences from the control female found in this specimen may be within the limits of individual variation resulting from these differences in zygotic sex balance. Only a careful study by biometric methods of a large number of single female embryos at this stage can settle this point. Such a study has not been made because of lack of material and the long time involved in search.

In any case, should this case fall inside the limits of individual variation discussed in the preceding paragraph, it will show all the more conclusively that the first stage of the freemartin is, at least, in some cases, a female, normal for that stage of development.

CONCLUSIONS.

1. On the basis of this 32 mm. freemartin and comparison with others described by the writer and by others, where the freemartin modifications are conditioned by vascular unions between the placentæ and not by mere contact and adhesion of one to the other, it is concluded that a blood-carried agent in the nature of a hormone is effective in producing the freemartin rather than an "organizer" of the type found in Spemann's work on early amphibian development.

2. Small vascular or capillary anastomosis is sufficient to condition the freemartin effects in some cases at least; but such small amounts of transfusion as are possible in such cases at first appear to delay the modifications and permit longer female development before the effective minimal concentration of hormone is reached on the female side. Early stages in such cases show a more markedly female embryo than do those with strong anastomosis, particularly in respect to gonad and müllerian ducts. Study of such borderline cases as these lend support to the "all-or-none" conception of the hormone action.

3. Cords of Pflüger or secondary sexcord regions develop frequently in freemartins and are resorbed later at unequal rates in different parts of the gonad, so that some of the peculiarly complicated interiors of gonads figured by previous students of the freemartin gonad can be interpreted in the light of these earlier stages as the results of survivals of isolated parts of the secondary sexcord region, separated from the medulla of the gland by tunica albuginea. Organization into sexcords may not be possible from the first and some individuals may not develop

cords though they may have the cell proliferations from which the cords would later develop but for the action of the hormone. This unequal development of sexcord region and unequal resorption seem to be related in some way to pressure by surrounding organs.

4. There is no intergrading in freemartins between the freemartin types and male types except in rare cases which resemble the anomalies found among single born males, where the sex balance appears to be upset. And in postnatal freemartins there is no intergrading to the female type. But in prenatal freemartins there is a definite series of intergrades to the female condition as the younger stages are studied till practically the female condition is reached. This furnishes the strongest of proof for the theory of the freemartin maintained by Lillie and by Keller and Tandler.

5. There are probably individual differences in zygotic sex balance among both male and female cattle. This is a factor in the variations of condition among freemartins and doubtless is operative from the bull's side as well as on the freemartin side. The hormone may not be equally potent in all cases and it may possibly differ in potency at different times in the same male, or be delayed in its appearance. In this way the hormone action may be delayed even when early complete anastomosis is effected. So, too, it is possible that the differences between this freemartin and the control female may be within the limits of individual variation correlated with zygotic differences in sex balance, for the exact limits of such variation have not yet been determined.

6. If so, it is even stronger proof for the theory of the freemartin of Lillie and of Keller and Tandler, since it would show that the first stage of the freemartin is a female, normal in sexual condition for that age.

SUMMARY.

1. A 32 mm. freemartin twin to a 34 mm. male is compared with a normal female of like size as to reproductive organs, and with normal male as to gonad.

2. It differs from the female control in having a thinner secondary sexcord region and thinner müllerian duct, lacking lumen in places where the control has one. Nevertheless it is more like

the female than any other freemartins with vascular anastomosis so far studied.

3. Placental vascular anastomosis between the twins was very slight—hardly more than capillary—and the placentæ pulled apart with slight bleeding. So it permitted very limited transfusion of male hormone, a situation correlated with slight modification from female type shown in small gonad and thin ovarian cortex and müllerian duct.

4. Comparison with other freemartins as to placental and sexual conditions indicates a blood-borne hormone as the agent rather than an "organizer" found effective in early amphibian differentiation. Mere tissue cohesion fails to condition the modifications as it does in Spemann's transplants.

5. Comparison with other gonads figured for freemartins and with new material indicates that some formerly anomalous regions in older freemartin gonads are derived from secondary sexcord remnants which failed to be resorbed and that these cortical regions frequently develop in freemartins to some degree and may or may not disappear.

6. This study adds support to the theory of the freemartin of Lillie and Keller and Tandler, that it is female, normally determined zygotically, but abnormally differentiated sexually by the intervention of a male hormone received from her twin brother in utero.

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ABBREVIATIONS.

Bl., bladder.
B. V. Gl., bulbovestibular or Bartholin's glands.
Clit., clitoris.
Gon., gonad.
Gr. Ph. Un., undercut groove in phallus.
Gub., gubernaculum.
Mesoneph., mesonephros.
Mül. Dt., müllerian duct.
Ost., ostium tubæ abdominale.
Rect., rectum.
Sac. Vag., saccus vaginalis.
Scr., scrotum.
Ura., urethra.
Vest., vestibule.
Vul., vulva.
W. Dt., Wolffian duct.

THE DIGESTIVE SYSTEM AND ITS FUNCTION IN *FUNDULUS HETEROCLITUS.*

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In the course of investigation of the function of the alimentary canal in *Fundulus heteroclitus* (North American common killifish, mudfish, mummichog[1]), it was found that this animal does not possess a stomach, *i.e.*, an organ secreting the pepsin-hydrochloric acid. Although several species of fishes do not possess peptic glands (see in Oppel's "Lehrbuch der Vergleichenden Mikroskopischen Anatomie der Wirbeltiere," 1 Teil, Jena 1896, S. 33, the corresponding table), we have never seen any reference to any investigation concerning the gastric digestion in *Fundulus heteroclitus.*[1] From the anatomical as well as from the physiological point of view, the digestive system of the *Fundulus* presents great peculiarities, which are worth attention. The present paper is to be regarded as a preliminary communication. A series of investigations were made by one of us (B. P. B.) during the summer of 1926 at the Atlantic Biological Station, St. Andrews, N. B., and subsequently one of us (D. J. B.) made histological examinations at the Department of Physiology, University of Toronto.

Anatomical Data.

The whole family of Pœciliidæ are comparatively small fishes. The largest specimens of *Fundulus* taken at Birch Cove on the Bay of Fundy, near St. Andrews, N. B., were from 65 to 83 mm.

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[1] It is interesting to note that the whole family of Cyprinidæ (*Cyprinus carpio,* etc.) are deprived of peptic glands. *Fundulus* belongs to the family of Cyprinodontidæ, and the Germans call it "Amerikanische Zahnkarpfen" (*Brunning* (2)). According to *Gill* (3) "the Cyprinodonts or Poecilids are really related to the Esocids and Umbrids, and to them they should be approximated in the sub-order Haplomi." The results of the present investigation, especially the absence of the glands secreting the pepsin-hydrochloric acid, show that *Fundulus* has some features in common with the Cyprinidæ.

in length, measured from the snout to the insertion of the caudal fin.

The alimentary canal in this species is comparatively short, being about equal to the length of the body, excluding the caudal fin. On opening the abdomen and carefully dissecting away the liver, we see a short œsophagus directly connected to the intestine.

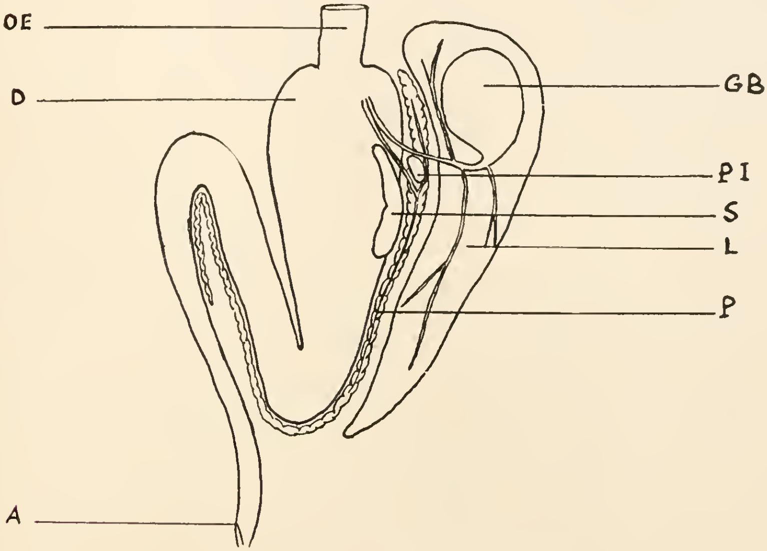

FIG. 1. Diagram of the alimentary tract of *Fundulus*. *Œ*, œsophagus; *D*, duodenum; *GB*, gall-bladder; *P.I.*, principal islands of Langerhans; *S*, spleen; *L*, liver; *P*, pancreas; *A*, anus.

Longitudinal sections through the junction of œsophagus and intestine show that, at this point, there is a well-developed sphincter quite comparable to the pyloric sphincter in mammals. The intestine is bent upon itself ventrally and to the right to form three portions; a first portion, descending; a second portion, ascending; and a third portion, descending to the rectum (Fig. 1). Pyloric cæcæ are not present.

The first part of the intestine, especially when distended with food, has a somewhat pear-shaped form. The second and third parts of the intestine do not display any such unusual degree of dilatation when food is present in them.

In Fig. 2 is represented a scheme of the alimentary tract of

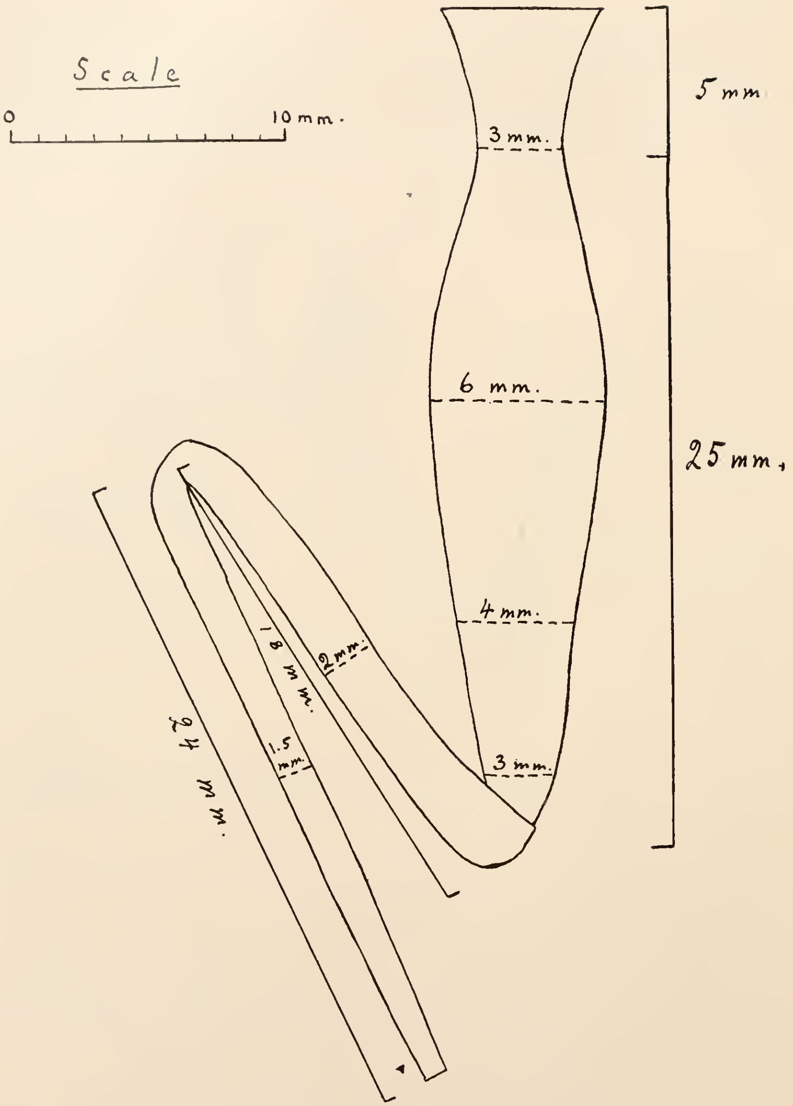
Scale
0
10 mm.
5 mm
3 mm.
6 mm.
25 mm.
4 mm.
3 mm.
2 mm.
18 mm.
1.5 mm.
24 mm.

FIG. 2. Scheme of the alimentary tract of *Fundulus*. The intestinal tract is fully relaxed.

Fundulus. The actual measurements are taken from a female *Fundulus* whose body length (caudal fin excluded) was equal to 78 mm.

The capacity of the first part of the intestine is four times greater than the capacity of the second part. Without pressure in the first part, four drops of water enter; in the second part only one drop enters. Under a moderate pressure (filling from a pipette) the cardiac part retains 7 drops, and 8 drops more may be introduced into it by using a greater pressure. Thus the arrangement of the initial part of the alimentary canal of *Fundulus* is similar to the so-called "syphonal" stomach of many other fishes. From a physiological point of view the first part of the intestine of the *Fundulus* fulfils one of the duties of the stomach, being *a receptacle and container for the food.*

In order to avoid repetition of cumbersome phraseology we shall frequently designate the first part of the intestine as the duodenum, since, as will be shown, it is into this part that the bile and pancreatic juice are poured. Both the physiological and the anatomical observations show that a stomach is really absent in *Fundulus.*

The liver is comparatively large, as it is in most fishes, and lies in a left ventral position, closely applied to the œsophagus and to the loop formed by the first and second portions of the intestine. In the upper part of the longitudinal space bounded by the liver and the three portions of the intestine, we find the gall-bladder, the spleen and the "principal" islets of Langerhans, along with a considerable portion of the diffuse pancreas. The pancreas has a wide distribution along the three parts of the intestine and is so diffuse that in fresh specimens it cannot readily be differentiated with the naked eye. The gall-bladder is comparatively large in these fish; in a specimen 78 mm. in body-length it measured 6 mm. by 3.5 mm., and had a capacity of 2.5 drops. The duct which drains the gall-bladder arises from the posterior end of that vesicle, but soon turns in an anterior direction and, after passing along the first part of the intestine to within 3 or 4 mm. of the œsophagus, it pierces the intestinal wall.

It is important to note that the pancreatic duct, though it runs parallel to the bile duct, does not unite with the latter to form a common duct, but has its own separate opening into the intestine

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beside the orifice of the bile channel. The hepatic ducts communicate with the cystic duct to form the common bile duct, though one or more of them may empty directly into the neck of the gall-bladder. The spleen is a small, flattened, bright red organ, and serves as a convenient landmark for locating the so-called principal islets of Langerhans, which are situated in the region between the neck of the gall-bladder and the duodenum. The largest of them, the " chief " principal islet, appears as a whitish, spherical nodule about 1 mm. in diameter. The pancreas is best studied in a series of stained sections. These show that slender processes extend off from the larger portions of the gland to invade the liver, particularly along the blood vessels and less so along the hepatic ducts. Sections show also that a thin layer of pancreatic tissue completely envelopes the gall-bladder. Serial sections made with the aim to study the duct connections of the pancreas which surrounds the gall-bladder showed that the ducts are leading away from this pancreatic tissue and communicating with the larger pancreatic ducts. It is important to note, however, that in no case were we able to find any evidence of communication between any of the channels for bile and any of those for pancreatic juice.

Methods.

Since the fistula method is not practical for the study of the digestive processes in fishes, after a suitable interval following natural or forced feeding the contents of the duodenum were sucked out by means of a pipette with a rubber bulb on the end. This method had the well known disadvantage that the intestinal secretions were mixed with food masses and also that removal of the partially digested food caused more or less disturbance of the normal process of digestion. *Funduli* are exceptionally hardy fishes and did not show any ill effects from the manipulations connected with the investigations of their intestinal contents.

The fish were kept in small aquaria or in glass jars containing running sea-water, or else in jars in which the water was kept cool (below 15° C.) and was well aërated. When kept in non-aërated sea-water which is allowed to reach a temperature of 15° to 20° C. (a comparatively high temperature for these *Funduli*), the fish very often eject the food through the mouth and some of them may die under such unfavorable conditions.

The reaction of the intestinal contents was first tested by means of litmus paper. Since the indications of litmus are not very reliable, whenever possible the hydrogen ion concentration was determined by the " spot " method elaborated by Felton (4). The values of the pH found in the present investigation must be looked upon as approximate only, because in most cases the intestinal fluids were mixed with food masses, a fact which would introduce the " protein " and " salt " errors described by Clark (5). The presence of bile in the intestinal contents was determined by Gmelin's test and by the surface tension test with flowers of sulphur. The methods employed for the detection of different enzymatic actions will be given later on.

As a preliminary measure all of the fish were deprived of food for one or more days prior to the experiments.

Experiments on Fasting *Funduli*.

Usually in fishes which have fasted for one or more days the duodenum is practically empty, it being difficult to obtain even as much as one drop of fluid in the pipette and what is obtained is composed partly of mucous. In some cases one may obtain two or three drops of a yellowish fluid which gives quite positive results in tests for bile, and occasionally the remains of copepods and other small animals may be found when microscopic examination of the contents is made. After 7 to 10 days of starvation, it is hard to get anything at all from the duodenum. Water is very seldom present in this part in fasting *Fundulus*.

The contents of the duodenum of fasting *Fundulus* is always alkaline to litmus, and its hydrogen ion concentration, as determined in more than 50 fishes ranges from pH 8.0 to pH 9.2. The variations in alkalinity depend chiefly on the presence or absence of bile, since bile taken directly from the gall-bladder has a hydrogen ion concentration of only pH 7.0 to 7.2. When mucus alone was present, the values were pH $=$ 8.8 to pH $=$ 9.2, whereas when bile also was present the values recorded were from pH $=$ 8.0 to pH $=$ 8.6. Regarding the possible presence and influence of sea water, we may say that samples taken from the sea-water pipe system at St. Andrews Biological Station were found in several determinations to have pH values of from 8.0 to 8.1. The contents of the second and third parts of the intestine of freshly killed fasting fish were also always alkaline to litmus.

Experiments on Fed *Funduli.*

Although *Funduli* prefer animal food such as clam and the internal organs of dead fishes, even of their own species, they are practically omnivorous and will eat fish muscles, raw meat, bread, hardboiled egg (yolk and white) and even watermelon. They digested perfectly well milk introduced by a pipette into the duodenum. The alimentary tract of freshly caught *Funduli* may contain mud, and small animals, insects, copepods, etc., but no matter what might be the nature of the food eaten or the stage of its

Table I.

Digestion of Clam in *Fundulus.*

Date.	Fish No.	Contents of Intestine.	Reaction of the Contents.	
			pH.	Litmus.
July 17:	1	Yellowish fluid	8.8	Strong alkaline
9.30 A.M.	2	" "	8.6	" "
to	3	" mucus	8.8	" "
10.00 A.M.	4	Mucus and water	8.6	Alkaline
	5	Mucus	8.8	Strong alkaline
10.15 A.M.		12 pieces of clam were put in the aquarium; 6 pieces were eaten at once. At 12.00 noon two pieces were left, which were removed from the aquarium		
12.15 P.M.	3	Pieces of clam; no bile	8.4	Alkaline
	4	Scant; no bile	8.8	Strong alkaline
2.15 P.M.	1	Clam; bile	8.2	Alkaline
	2	" "	8.8	"
	3	Scant; "	8.6	"
	4	Nil.	8.6	"
	5	Clam; bile	8.4	"
7.15 P.M.	1	Nil.	8.4	"
	2	Clam; bile	8.2	"
	3	Mucus only	8.8	Strong alkaline
	4	" "	9.0	" "
	5	Clam; a little bile	8.8	" "
July 18: 9.00 A.M.	5	Yellowish mucus	9.2	" "

digestion, the reaction in the duodenum as well as in the second and third parts of the intestine was always alkaline, both in fish that were caught and investigated at once, on the spot, and also in those kept in aquaria and fed naturally or forcibly. However, the degree of alkalinity varied somewhat, according to the nature of the contents. As stated above, the reaction in the duodenum of fasting *Funduli* is decidedly alkaline, pH = 8.0 to 9.2. The presence of foods rich in protein, such as meat and clam, which are acid; of milk, which is neutral or only very slightly alkaline; of sea-water (pH = 8.2); and particularly the presence of considerable amounts of bile, which is also nearly neutral (pH = 7.0 to 7.2), all tend to reduce the degree of alkalinity.

Table I shows the course of digestion in *Fundulus* after natural feeding with clam. The contents of the duodenum were sucked out at certain intervals for each of five fishes. Prior to feeding they had been fasting for five days, and no excrements were found in the aquarium during the last two days of this period. Shortly before the fast was terminated, the pipette was introduced into the duodenum to withdraw any fluids which might be present.

Results analogous to those shown in the table were obtained with all the other kinds of food; the reaction in the duodenum was always alkaline.

The following experiment shows that there is an active discharge of bile into the intestine after a meal has been swallowed.

Experiment of August 4.

In an aquarium in which nine fishes were kept for several days without food, at 8.30 A.M. there were placed several pieces of clams' flesh, but the amount was not sufficient to feed all of them. At 8.30 P.M. all of these fishes were killed and the contents of the duodenum and of the gall-bladder were investigated. In five fishes the duodenum was filled with clam and bile, while the gall-bladder was quite empty and collapsed. In the other four fishes the duodenum was empty, while the bladder was distended with bile.

In the case of injection of milk into the duodenum, the digestion was also always alkaline, the degree being somewhat less in the first hours (pH = 7.8) than in the later hours (pH = 8.8). Bile was present in the contents. The pH of the milk itself was pH = 7.2.

Alkaline reaction was also always found during the digestion of raw beef introduced into the duodenum. Six and one half hours after forcible feeding the contents were sucked out and the reddish-yellow fluid obtained gave an alkaline reaction, pH = 8.2.

Reaction to Various Stimuli.

Mechanical Stimulation.

Experiment of July 10.—As a means of mechanical stimulation, a few small pieces (2 mm. cubes) of cork or rubber were introduced into the duodenum in three *Funduli,* which were starved for at least three days. Just prior to the experiment the contents of the duodenum, consisting of a very little amount of mucus and some bile, were tested and found to be alkaline, pH = 8.6 to 9.0.

At 10.15 A.M., three pieces of cork were introduced into the duodenum of one fish; two pieces into that of the second; and two pieces of rubber into that of the third fish. The duodenal contents were sucked out at 2.15 P.M. and at 6.15 P.M. At both times, in each case, one could remove from 2 to 4 drops of water, with some mucus, but in only one fish was bile present, as indicated by the slightly yellowish colour of the contents. The reaction was in all cases alkaline, pH = 8.2 to 8.6. The next morning the corks were found in the aquarium, stuck together with mucus and probably had been ejected through the anal opening.

It is interesting to note that towards the end of the experiment water was found in the duodenum of these fishes, since its presence is quite unusual. From these experiments it appears that purely mechanical stimulation of the duodenum does not provide the necessary stimulus to provoke the pouring of bile into the intestine but that it does cause the fish to " drink water."

Hydrochloric Acid.

Table II, gives the results of introducing a few drops of 0.36 per cent. hydrochloric acid into the duodenum in two fishes that had been starved for 10 days. Its introduction did not provoke a flow of bile into the gut.

The table shows that in 6 hours after the introduction of 0.36 per cent. hydrochloric acid the duodenal contents had again become alkaline. It is interesting to note that in warm blooded animals the introduction of hydrochloric acid in the concentration in which

TABLE II.

INTRODUCTION OF HCl INTO DUODENUM.

Date and Time.	Fish No.	Contents of Intestine.	pH.	Litmus.
August 13: 9.00 A.M.	1	Almost nil.	—	Alkaline
	2	" "	—	"
9.10 A.M.		Introduced 5 to 6 drops 0.36% HCl		
10.10 A.M.	1	Mucus and fluid	4.8	Acid
	2	" " "	5.0	"
11.10 A.M.	1	Mucus; very little fluid [1]	5.0	"
	2	" " " " [1]	5.4	"
12.10 P.M.	1	Mucus only [1]	5.0	"
	2	" " [1]	5.4	"
3.10 P.M.	1	Mucus and water	7.8	Very weak alkaline
	2	Watery fluid	7.6	" " "

it is in the gastric juice, *i.e.,* 0.5 to 0.36 per cent. practically does not provoke the entry of bile into the duodenum.

Alcohol.

From 4 to 6 drops of 5 per cent. pure ethyl-alcohol was introduced into the duodenum by means of a pipette 3 to 4 times per day. Four sets of analogous experiments were performed. In one of them, 2 fishes received alcohol regularly during 6 days. In the earlier experiments, in order to make sure that the alcohol reached the duodenum, it was mixed with a little finely ground carmin and in every case the red granules were afterwards found in the duodenal contents and also in the excrements.

The results of one of these experiments are shown in Table III. The fish had been starved for 2 or 3 days before the experiment, and the duodenum was almost empty. The very small amount of fluid present was alkaline to litmus. When the duodenum was evacuated 2 or more hours after injection of alcohol, it was found to contain only a very small amount of fluid, usually mucus. Except occasionally in the morning tests after a night when the fish did not receive injections of alcohol, there was no bile present in the duodenum. The alkalinity of the contents was very high, pH = 8.8 to 9.2, but it was not determined whether this was due to the presence of pancreatic juice or of intestinal juice. The alcohol injected was slightly acid, pH = 6.8.

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[1] To determine the reaction in these cases a few drops of distilled water were added to extracted material.

Table III.

Introduction of Alcohol into the Duodenum.

Date and Time.	Contents of Intestine.	pH.	Litmus.	5% Alcohol Injected.
July 19:				
4.30 P.M.		—	—	4 drops
7.00 P.M.	1 drop of fluid	8.6	Alkaline	" "
July 20:				
9.30 A.M.	Mucus	9.0	"	" "
2.45 P.M.	2 drops	8.8	Strongly alkaline	" "
7.00 P.M.	2 drops	8.8	Alkaline	" "
July 21:				
11.25 A.M.	1 drop and mucus	8.2	Alkaline	" "
12.25 P.M.	3 drops	8.8	Strongly alkaline	" "
3.30 P.M.	2 drops	9.0	" "	" "
7.15 P.M.	2 drops	9.0	" "	None
July 22:				
11.50 A.M.	Mucus only[1]	9.4	" "	4 drops
July 23:				
9.45 A.M.	Nil.	—	—	" "
11.50 A.M.	3 drops	8.8	Strongly alkaline	" "
2.45 P.M.	1 drop and mucus	9.0	" "	" "
July 24:				
9.15 A.M.	Mucus only[1]	9.2	" "	" "
12.15 P.M.	1 to 2 drops	9.2	" "	None

N. B. Bile was not found in the contents during this experiment.

It is important to note that the experiments with alcohol afford further evidence that gastric glands are absent in Fundulus. Although alcohol is one of the strongest stimuli for the secretion of gastric juices, in our experiments the intestinal contents never had an acid reaction, but, as stated above, were always highly alkaline.

Pilocarpin.

Table IV. gives the results of one of eight sets of experiments with pilocarpin. Five or 6 drops of 1 per cent. solution of pilocarpin was injected into the duodenum of two fishes which had been starved for 7 days. At the end of this period the duodenum contained in each case a small amount of mucus and fluid which was strongly alkaline, pH = 9.2 and 9.0 respectively.

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[1] To determine the reaction in these cases a few drops of distilled water was added.

The interesting feature of the experiments is that pilocarpin provoked chiefly the motor phenomena of the alimentary canal. Its introduction into the duodenum excited evacuation of the bowel and this was accompanied by the entry of the bile into the intestine. The contents of the duodenum, extracted one hour after injection of the pilocarpin, had a yellowish or greenish colour and gave a positive test for bile, with flowers of sulphur. Following the injection, the alkalinity of contents of the duodenum was comparatively low, pH between 7.8 and 8.6.

TABLE IV.

INTRODUCTION OF PILOCARPIN INTO THE DUODENUM.

Date and Time.	Contents of Intestine.	pH.	Litmus.
August 10:			
8.35 A.M.	1. Mucus and some fluid	9.2	Alkaline
	2. " " " "	9.0	"
8.45 A.M.	Injected 5 to 6 drops of 1% sol. of pilcarpin into each fish		
9.45 A.M.	1. 2 to 3 drops of emerald green bile	8.0	Weakly alkaline
	2. 8 drops of green fluid; re-injected 6 drops of it	7.8	" "
10.45 A.M.	1. 2 to 3 drops of light green fluid	8.4	" "
	2. 10 drops of green fluid; re-injected 8 drops of it	8.2	" "
11.45 A.M.	1. 2 to 3 drops of light green fluid	8.4	Alkaline
	2. 8 drops of green fluid	8.2	Weakly alkaline
3.15 P.M.	1. 4 to 5 drops of a very light green fluid with mucus	8.6	Alkaline
	2. 3 to 4 drops of clear, colorless fluid	8.6	"

Atropin.

The injection of 1 per cent. atropin sulphate into the duodenum, as one would expect, inhibited the intestinal secretions (it was hard to get anything but mucus from the intestine) and also prevented the entry of bile into the gut. Table V. gives the results of experiments on two fishes which had been starved for 10 days.

The reaction of the duodenal contents was usually strongly alkaline.

It is interesting to note that one hour after the introduction of atropin into the intestine, the skin of the fish became dark, almost black. In five hours the normal colour of the skin had returned. These changes were not due to light effects, as the whole day was grey and cloudy.

From the foregoing experiments it may be seen that the first part of the alimentary canal of Fundulus may properly be designated the duodenum, since it corresponds to the duodenum of vertebrates possessing a stomach.

TABLE V.

INTRODUCTION OF ATROPIN INTO THE DUODENUM.

Date and Time.	Contents of Intestine.	pH.	Litmus.
August 13:			
9.10 A.M.	1. Nil. 2. "	— —	Alkaline "
9.20 A.M.	Injected 4 to 5 drops of 1% sol. of atropin sulphate into each fish		
10.20 A.M.	1. 2 to 3 drops of fluid, with mucus 2. 1 drop of fluid, with mucus	9.0 8.8	Strongly alkaline Alkaline
11.20 A.M.	1. Very little fluid and mucus; difficult to obtain it 2. Very little fluid and mucus; difficult to obtain it	8.8 8.8	" "
12.20 P.M.	1. Almost nil.; a little mucus 2. Almost nil.; a little mucus	8.8 8.8	Strongly alkaline Alkaline
3.20 P.M.	1. Mucus and 1 to 2 drops of water 2. Mucus and 3 drops of water	9.0 8.8	Strongly alkaline " "

N. B. There was no bile present in the intestinal contents during the whole experiment.

ENZYMES OF THE MUCOUS MEMBRANE OF THE FIRST PART OF THE INTESTINE.

The only method which we applied to the study of the enzymes of this mucous membrane was the reaction of its extract on various media. The imperfections of this method are very well known. A certain possibility that we might have to consider autolytic enzymes, as well as digestive enzymes, was suggested by the observations of Bradley (1922[6]), on autolysis of liver and kidney. He produced evidence to show that in autolysis there are two autolytic enzyme-complexes, one of which is analogous to trypsin, but has its optimum activity at pH 4 to 4.5, whereas true trypsin has its optimum activity at pH 8. Beyond the range from pH 7 to pH 3, the action of this autolytic enzyme-complex is insignificant, while for trypsin the range of activity is pH 9 to pH 4.5. The other autolytic enzyme-complex is analogous to pepsin, but has its optimum reaction at pH 4.5, whereas pepsin has its optimum at pH 1.5. It is destroyed at pH 2.6, while pepsin is very active at that degree of acidity. The range of activity for pepsin is from pH 0.5 to pH 6.5 (McFarlane, et al.[7]).

By adjusting the reaction of the media to hydrogen ion concentrations which are beyond the limits of activity for any autolytic enzyme-complexes which might be present, one could expect to obtain reactions due solely to digestive enzymes. To minimize the possibilities of contamination all extracts of the intestinal mucous membrane were kept under toluene and all test tubes and pipettes were sterilized before use.

The extracts from the mucous membrane usually of the duodenum were prepared in the following way. The intestine, cut out from the body, was cleaned from all surrounding tissues, split longitudinally, and washed several times in tap water. Only the mucous membrane was scraped by a knife and mixed with the corresponding fluid. The temperature in the incubator was 35° C.

For use in the various experiments extracts of the mucous membrane of the intestine were made with 30 per cent. alcohol, 0.9 per cent. NaCl, glycerine, and 0.36 per cent. HCl. These were used at various intervals after their preparation and were kept under various conditions of temperature.

In preparing the extracts for use they were filtered, or otherwise separated from the tissue cells, and were then diluted with 3 or 4 volumes of distilled water. The hydrogen ion concentration of the mixture was adjusted to the desired level by adding either 0.36 per cent. HCl or dry sodium carbonate. All tests of enzymatic action reported here must be looked upon as qualitative only.

Action of the Extracts on Protein (Fibrin).

For the experiments on the digestion of protein, fibrin was extracted from fresh blood and was then kept in glycerol. Before use it was thoroughly washed; dried between leaves of blotting paper; and then minced and small portions of it were placed in several test tubes along with the extract to be tested and a few drops of toluene. The whole mixture was incubated at 35° C.

In order to rule out the problem of autolysis the reaction of the media during these tests was adjusted to H ion concentrations of pH = 2; pH = 8.0; pH = 8.4 and pH = 9.0 respectively.

No trace of digestion of fibrin was manifested in periods ranging from 48 to 62 hours respectively, a fact which indicates that the extract of intestinal mucous membrane contains neither pepsin nor trypsin.

In one case, on the sixth day the fibrin was found to be partly dissolved. The initial pH of the mixture in this case was 8.4, but it may have been that during such a long period of incubation the medium may have lost its alkalinity to such an extent that the autolytic enzyme-complexes had started to act.

Action of the Extracts on Peptone.

In one set of experiments a very gradual but not complete discoloration of a mixture of purified casein, phenol red, and saline extract was observed during 24 hours in the incubator. The initial pH of the mixture was 8.0.

In another set of experiments a 2 per cent. solution of Witte's peptone was mixed with boiled, and with unboiled, saline intestinal extract, respectively, and was adjusted to pH = 7.8. After 3 days in the incubator biuret tests showed that there was less peptone in the mixture with fresh extract, than in the control, in which boiled extract was used, indicating that the fresh extract had a weak ereptic action.

Action of the Extracts on Starch.

Table VI. gives the results of two experiments in which the extract of the intestinal mucous membrane on normal saline was allowed to act on starch. In one case fresh extract was used and in the other the extract was boiled. In each case the mixture was adjusted to pH 6.7 and 2 drops of toluene were added, after which it was placed in the incubator. In the subsequent test for starch a weak solution of iodine in potassium iodide was employed. The table shows that, whereas the tests with boiled extract were negative, those with fresh extract gave a progressive diminution in the amount of starch present and a corresponding increase in the sugar content of the mixture, indicating a decided amylolytic action.

That the amylolytic action of the intestinal extract was due to the enzyme contained in the mucous membrane and not to the pancreatic juice, which could be fixed on the intestinal mucous membrane, in spite of its thorough washing, is seen from the fact that proteolytic action was absent in the same extracts.

TABLE VI.

AMYLOLYTIC ACTION OF EXTRACT OF THE INTESTINAL MUCOUS MEMBRANE.

Date.	Experiment 1.	Experiment 2.
August 4......	3 cc. of 2% soluble starch 10 drops boiled extract pH = 6.7 Iodine reaction—blue	3 cc. of 2% soluble starch 10 drops fresh extract pH = 6.7 Iodine reaction—blue
3.30 P.M.....	Placed in incubator	Placed in incubator
August 5:		
8.30 A.M.....	Iodine—no change Fehling—negative pH = 6.7	Iodine—light purple Fehling—positive pH = 6.7
7.30 P.M.....	Iodine—no change	Iodine—light purple
August 6:		
10.15 A.M.....	Iodine—no change Fehling—no change pH = 6.6	Iodine—very light purple Fehling—strongly positive pH = 6.7
7.30 P.M.....	—no change	—progressive change
August 7:		
7.30 P M.....	Iodine—no change Fehling—no change pH = 6.6	Iodine—almost colorless Fehling—strongly positive pH = 6.7

Action of the Extracts on Lipoids.

It was found that unboiled extract of the intestinal mucous membrane had a weak lypolytic action on olive oil and a somewhat more pronounced effect on cream. The reaction on cream is shown in Table X., experiments 5 and 6.

Enterokinase of the Extract.

In addition to its action as a ferment acting directly on peptones, starch and fats, it was shown that the unboiled intestinal extract had also the power of greatly accelerating the tryptic action of liver extract of *Fundulus* and of the bile: to a lesser degree, this extract increased the action of lipase on the digestion of fat, whereas boiled extracts had no such effects.

Activation of Trypsinogen.

Table VII. shows the results of adding unboiled saline intestinal extract to a mixture containing minced fibrin and glycerin extract

Table VII.

Activation of Trypsinogen by Extract of Intestinal Mucous Membrane.

Date.	Exp. 1.	Exp. 2.	Exp. 3.
August 13:			
12.00 Noon	Minced fibrin 8 dr. glycerin extract of hepatic pancreas 12 dr. water No intestinal extract (control) pH = 8.2	Minced fibrin 8 dr. glycerin extract of hepatic pancreas 10 dr. water 2 dr. intestinal extract (in saline) pH = 8.2	Minced fibrin 8 dr. glycerin extract of hepatic pancreas; and intestinal mucous membrane prepared a day before 12 dr. water 2 dr. intestinal extract (in saline) pH = 8.2
1.15 P.M.	No change	No change	Completely digested
3.30 P.M.	" "	Digestion begun	—
5.30 P.M.	" "	Half digested	—
6.30 P.M.	" "	Completely digested	—
August 14:			
9.00 A.M.	" "	—	—
August 15:			
9.00 A.M.	" "	—	—
August 16:			
8.30 A.M.	Marked digestion	—	—
1.00 P.M.	Half digested	—	—
3.00 P.M.	Completely digested	—	—
			N.B. In this experiment (3) the extracts of hepatic pancreas and of intestine were mixed together on the previous day

of the liver of *Fundulus*. (As stated above, the liver of these fishes is invaded by pancreatic tissue, so that extracts might contain considerable trypsinogen.) In Experiment No. 3, the fibrin was very quickly digested. The probable cause was that the liver extract and the extract of the intestine had been mixed together on the previous day and that in the interval the trypsinogen had already become active trypsin. In Experiment No. 2, the activation of protrypsin required a latent period of about 3 hours. The presence of glycerin in the mixture probably inhibited the process of activation. In Experiment No. 1, intestinal extract was not used, and, therefore, digestion did not take place until the third day. The most probable explanation of this phenomenon is that it required such an interval for the spontaneous activation of the protrypsin.

Other experiments which demonstrate the activation of trypsinogen by intestinal extracts are recorded in Table IX.

Acceleration of Lipolysis.

The experiments in which the digestion of fat was accelerated by the addition of unboiled intestinal extract to a mixture containing bile and cream are recorded in Table X. and in Table XI.

Enzymes in Gall-bladder Bile.

At the time of these experiments, the histological investigations had not been made and, therefore, it was not then known that the gall-bladder of *Fundulus* is surrounded by a thin layer of pancreatic tissue, the presence of which introduces the possibility that a small amount of pancreatic enzyme may have become mixed with the bile during the manipulations for emptying the gall-bladder.

The gall-bladder was dissected out and removed from the body with sterile instruments, the bile was pressed out of the viscus, and the contents were collected in a sterile container. The bile secured in this manner contained a very active amylase, trypsinogen and prolipase. The last two substances could be activated by the unboiled extract of intestinal mucous membrane.

Table VIII. gives the results of two experiments to demonstrate the amylolytic action of fresh bile.

TABLE VIII.

AMYLOLYTIC ACTION OF GALL-BLADDER BILE.

August 7:

Time.	Experiment 1.	Experiment 2.
9.30 A.M.	2 cc. of 2% soluble starch 2 drops boiled bile pH = 6.6	2 cc. of 2% soluble starch 2 drops fresh bile pH = 6.6
9.40 A.M.	Placed in water bath at 35° C.	Placed in water bath at 35° C.
10.40 A.M.	Iodine—dark blue Fehling—negative	Iodine—purple color Fehling—positive
11.40 A.M.	No change	Iodine—light purple Fehling—strongly positive
2.40 P.M.	No change	Iodine—very light purple almost colorless Fehling—strongly positive
	pH = 6.6	pH = 6.6

TABLE IX.

TRYPSINOGEN OF THE BILE AND ITS ACTIVATION BY THE EXTRACT OF THE INTESTINAL MUCOUS MEMBRANE.

Date and Time.	Experiment 1.	Experiment 2.	Experiment 3.
August 5:	Minced fibrin 2 cc. bile 8 cc. dist. water No extract (control) pH = 8.3	Minced fibrin 2 cc. bile 8 cc. dist. water 1 drop boiled intestinal extract pH = 8.3	Minced fibrin 2 cc. bile 8 cc. dist. water 1 drop fresh intestinal extract pH = 8.2
10.20 A.M.	Put in incubator	Put in incubator	Put in incubator
12.05 P.M.	No change	No change	Digestion begun
1.15 P.M.	" "	" "	Half digested
2.00 P.M.	" "	" "	Completely digested
7.30 P.M.	" "	" "	—
August 6: 10.20 A.M.	" "	" "	—
	Added 1 drop fresh intestinal extract	Added 1 drop fresh intestinal extract	
11.30 A.M.	Digestion begun	Digestion begun	
12.05 P.M.	Half digested	Half digested	
1.00 P.M.	Completely digested	Completely digested	

Table IX. shows that the bile alone or in the presence of boiled intestinal extract has no digestive action on fibrin, but when fresh

TABLE X.

PROLIPASE IN GALL-BLADDER BILE LIPASE AND ENTEROKINASE IN EXTRACTS OF INTESTINAL MUCOUS MEMBRANE.

Date.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
Aug. 12	Cream substrate (control)	Cream substrate 4 dr. bile	Cream substrate 4 dr. bile 2 dr. fresh intestinal extract	Cream substrate No bile 4 dr. boiled intestinal extract	Cream substrate No bile 4 dr. fresh intestinal extract
3.00 P.M.	Put in incubator	Put in incubator	Put in incubator	Put in incubator	Put in incubator
4.30 P.M.	No change	Light purple 1 dr. NaOH added	Light green 2 dr. NaOH added	No change	No change
6.00 P.M.	" "	Purple	Light green 3 dr. NaOH added	" "	" "
8.00 P.M.	" "	Light green 1 dr. NaOH added	Light purple 1 dr. NaOH added	" "	Light pink
Aug. 13: 8.00 A.M.	" "	Light green 3 dr. NaOH added	Grey 2 dr. NaOH added	" "	White 1 dr. NaOH added
3.00 P.M.	" "	Grey-purple 1 dr. NaOH added	Pink-purple 1 dr. NaOH added	" "	Pink
NaOH solution added during 24 hours	Nil.	6 drops	9 drops	Nil.	1 drop

TABLE XI.

PROLIPASE IN THE GALL-BLADDER BILE, ITS ACTIVATION BY THE INTESTINAL EXTRACT.

Date.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
Aug. 16	Cream substrate (control)	Cream substrate 5 dr. of boiled bile	Cream substrate 3 dr. of fresh bile	Cream substrate 2 dr. boiled bile and 2 dr. boiled intestinal extract	Cream substrate 2 dr. of fresh bile and 2 dr. of fresh intestinal extract
11.00 A.M.	Put in incubator	Put in incubator	Put in incubator	Put in incubator	Put in incubator
12.00 Noon	No change	No change	Light pink 1 dr. NaOH added	No change	Light pink 1 dr. NaOH added
1.00 P.M.	" "	" "	No change	" "	Light pink 1 dr. NaOH added
2.00 P.M.	" "	" "	Very little change	" "	Grey 1 dr. NaOH added
5.00 P.M.	" "	" "	No change	" "	Grey 3 dr. NaOH added
8.30 P.M.	" "	" "	" "	" "	Grey 3 dr. NaOH added
Aug. 17: 8.00 A.M.	" "	" "	Grey purple 1 dr. NaOH added	" "	Light pink 2 dr. NaOH added
NaOH solution added during 17 hours	Nil.	Nil.	2 drops	Nil.	11 drops

extract is used, tryptic digestion takes place in a short time. Apparently trypsinogen was present in the bile, and was activated by the fresh intestinal extract.

To these findings might also be added the fact that when toluene is not used, self activation of the tryptic enzyme in the bile takes place after one or two days at room temperature, and much more quickly in the incubator.

Tables X. and XI. show that the gall-bladder bile contains a prolipase, which may be activated by the extract of the intestinal mucous membrane. From the same tables we learn too that the intestinal extract possesses a weak lipolytic action and contains an activator for the bile lipase.

In all of the experiments recorded in Tables X. and XI. boiled cream, diluted with one volume of distilled water was used as the substrate, and to this was added one drop of 1 per cent. phenolphthalein and sufficient 0.09 per cent NaOH solution (2 to 4 drops) to produce in each case a distinct pink color (pH = 8.3). This alkaline pink mixture of diluted boiled cream is referred to in the table as the cream substrate. After decoloration of the mixture in the incubator, a sufficient amount of 0.09 per cent. NaOH solution was added to restore its initial pink color.

From Tables X. and XI. one may see that: the bile alone possesses a weak lypolitic action which develops slowly (Exp. 2, Table X. and Exp. 3, Table XI.); this action is due to an enzyme, because boiled bile loses its lypolitic action (Exp. 2, Table XI.); the intestinal extract alone has a weak lipolytic action (Exp. 5, Table X.); boiled intestinal extract loses its enzymatic action (Exp. 4, Table X.); when bile and intestinal extract are combined the lipolysis is much accelerated (Exp. 3, Table X. and Exp. 5, Table XI.); boiling destroys the lipolytic action and the activation (Exp. 4, Table XI.).

It is evident that the result of combined action of the bile and of the intestinal extract is not merely a simple summation of their activities, but indicates rather that the intestinal extract possesses a substance which activates the bile viz. pancreatic lipase. There is also a possibility that the bile increased the action of the intestinal lipase.

Since the presence of pancreatic tissue on the gall-bladder makes it possible that enzymes of the pancreas may have become mixed with the bile used in these experiments, the latter must necessarily be repeated with bile obtained by more adequate methods. If it can be demonstrated that the enzymes in question were admixed to the bile with the pancreatic cells surrounding the gall-bladder in *Fundulus,* these experiments will offer a proof that the pancreas of this animal elaborates the same enzymes as the pancreas of warm-blooded animals, two of them in form of zymogen (protrypsin and prolipase), and one in active form (amylase).

Discussion.

The data reported in this investigation show that *Fundulus heteroclitus* lacks a stomach, the intestine joining directly to the œsophagus. Since there are several species of fishes with an analogous structure of the alimentary canal, from the point of view of their digestive system, all fishes may be divided into two main groups, namely, (1) those possessing a pepsin-hydrochloric acid digestion, and (2) those lacking it. This makes it possible to differentiate two types of digestion in fishes; namely, acid-alkaline, and exclusively alkaline. There is very little doubt that these decidedly different types of digestion greatly affect the internal chemisms of the body, in the two cases; because, in the one case, important changes in the chemical composition of the body fluids are provoked following the secretion of acid into the stomach. Similar changes must necessarily be quite absent in fishes deprived of a stomach and must be greatly attenuated in the pathological syndrome, known as achylia gastrica, or in animals with experimentally removed stomachs. Therefore, the study of the animals of a type of *Fundulus* presents an interesting problem.

Summary.

1. That *Fundulus heteroclitus* does not possess a stomach is shown by the following evidence:

(*a*) There is absolute absence of pepsin and hydrochloric acid in the digestive juices.

(*b*) Every phase of digestion takes place in an alkaline medium.

(*c*) The first part of the intestine (duodenum) is joined directly to the œsophagus.

(*d*) The bile and pancreatic juice are poured into the gut only a few millimeters (3 or 4) below the œsophagus.

(*e*) The duodenum is capable of dilating to form a container for food.

2. Extracts of the intestinal mucous membrane manifest an amylolytic, a lipolytic and an ereptic digestive action. They probably contain also an enterokinase.

3. The gall-bladder bile contains a trypsinogen, a prolipase and an amylase. The origin of these enzymes must be investigated further, because they could be admixed to the bile from the layer of pancreatic tissue surrounding the gall-bladder, during its collection.

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GRAFTING AND REINCORPORATION IN ACTINOSPHÆRIUM EICHHORNII EHR.

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It is a well known fact that contiguous individuals[1] in a culture of *Actinosphærium eichhornii* may fuse along the surfaces in contact. Cytoplasmic union of this type, or plastogamy,[2] has been frequently described, and has often been considered as an important initial step in the evolution of nuclear union.

In the closely allied form *Actinophrys sol*, plastogamy is a preliminary step toward nuclear fusion. A striking photomicrograph of this process, taken from living material, is shown by Bělař, '22, (Taf. 3, Fig. 24). Fusion of a number of these individuals around a large food mass, has recently been described by Looper, '25. This union is temporary, for separation occurs as soon as digestion is completed.

Attempts to produce plastogamous fusion artificially have met with varying degrees of success. Cienkowski, '65, was able to fuse two vegetative masses of *Actinosphærium* if he brought together two cut surfaces, and held them together with paper strips. Johnson, '94, could not bring about artificial coalescence in a single instance. Greff, '67, and Penard, '04, observed that a single vegetative mass, if fragmented into a large number of portions under a cover slip, might eventually fuse into the original form. In some cases, this may have been a simple contraction phenomenon, for no attempt was made to break apart the cytoplasmic threads connecting the crushed fragments.

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[1] In behavior, the single sphere commonly termed an individual *Actinosphærium*, shows all the characteristics of a colonial aggregate. This would seem the more accurate terminology to apply to each vegetative mass.

[2] Also spelled plasmogamy. Bělař, '22.

In the course of an extended physiological study of Actinosphærium eichhornii, it was observed that artificial plastogamy could invariably be induced by suspending two or more animals in a hanging drop small enough to exert moderate compression. Various types of simple grafts were found to be successful when sufficient pressure was exerted by this method to enforce temporary contact.

Also, the interesting behavior of axopods[3] which were occasionally severed from the main body during the transfer of animals to hanging drops, seemed worthy of critical attention. Reincorporation of pseudopodial fragments has been reported in a few instances. Jensen, '96, and '01, describes this phenomenon in *Orbitolites* and *Amphistegina*. Kepner and Reynolds, '23, observed in *Difflugia* the recovery of separated pseudopodial fragments by their cell bodies.

The results derived from these accidental and experimental graftings, and from the severing of axopods, in *Actinosphaœrium* are presented in the present paper.

Material and Methods.

Actinosphœrium eichhornii was reared in large numbers in a medium containing ciliates, rotifers and round worms. Subcultures were made in Great Bear Spring Water, to which had been added concentrations of *Colpoda, Paramecium, Noteus* and *Philodina.*

Gross merotomy was performed under a binocular microscope. To aid in differentiating between the cut portions of two individuals, half of the animals to be grafted were stained in a very dilute solution of neutral red, made up in the original medium. The neutral red penetrated easily, and stained the cytoplasmic granules a deep red. A stained (red) and an unstained (white) animal were placed in a Syracuse watch glass, and cut into halves or quarters with a glass hair. Before normal contraction could cause rounding of these portions, two or more were transferred to a hanging drop, the size of which was reduced until the animals came into contact at the desired angle. These were transferred to a moist chamber for observation under high power (Leitz oc. 8x, obj. 7a).

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[3] Greeff (*loc. cit.*) saw "unipolar, pear-shaped cells," among the fragments resulting from his crushing experiments. In all probability, these were derived from axopodia, but their origin could not be accurately observed when so drastic a method of separation was applied.

Severing of individual axopodia was accomplished by means of the Chambers micrurgical apparatus, and the subsequent behavior followed under high power.

Results.

(*a*) *Grafting of Cut Portions.*

Grafted animals fused perfectly in all cases. The portions taken from each of the two contributing animals remained distinctly marked off. The surfaces held in contact varied in position and extent.

Record 3, (Text-figures 1 to 7), covers briefly the range of possible variations. In Figs. 1 and 2, two cut medullary surfaces (*M–M*) were brought into contact. Two quadrants of a stained individual brought into contact, medulla to medulla, (*M–M*), and medulla to cortex, (*m–C*), are shown in Figs. 3, 4 and 5. Figs. 6 and 7 represent a case in which an entire stained (red) animal was grafted on to half of an unstained (white) individual.

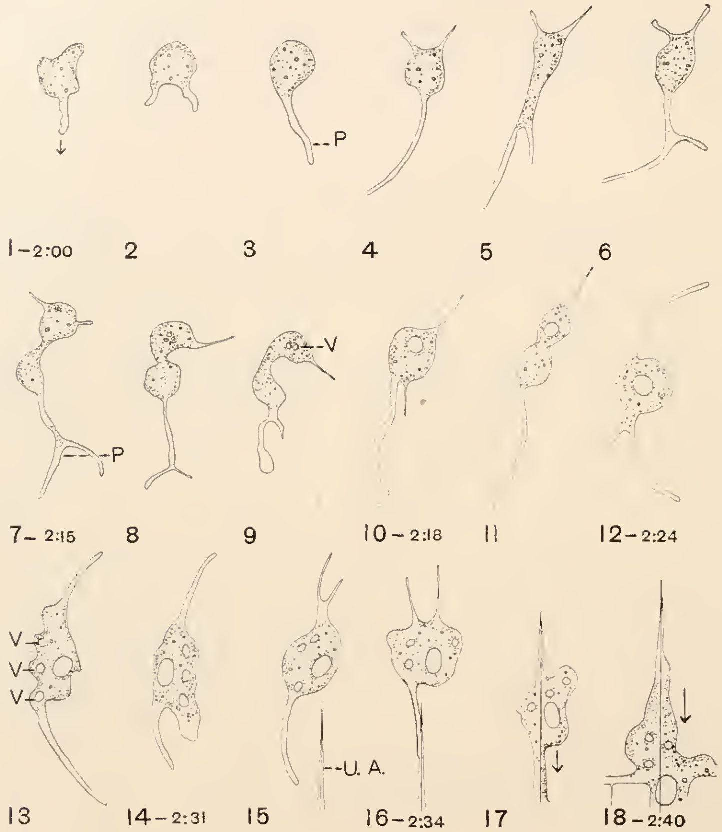
The time taken for complete fusion of surfaces varied from five to twenty minutes. As the animals were returned to the deeper water in a Syracuse watch glass, they resumed their normal spherical shape.

The extent and position of the grafted portions was still recognizable at the end of thirty hours.

(*b*) *Merotomy and Reincorporation of Axopodia.*

Axopodia were severed from the main body, carried away by the needle for some distance to break all connecting strands of protoplasm, and returned to the immediate region of the animal. Freed from the microneedle, they exhibited slow, erratic, swinging movements. If these movements brought them into chance contact with an unsevered axopod, or with the cortical surface of the main body, they were incorporated (Records 1, 2 and 5).

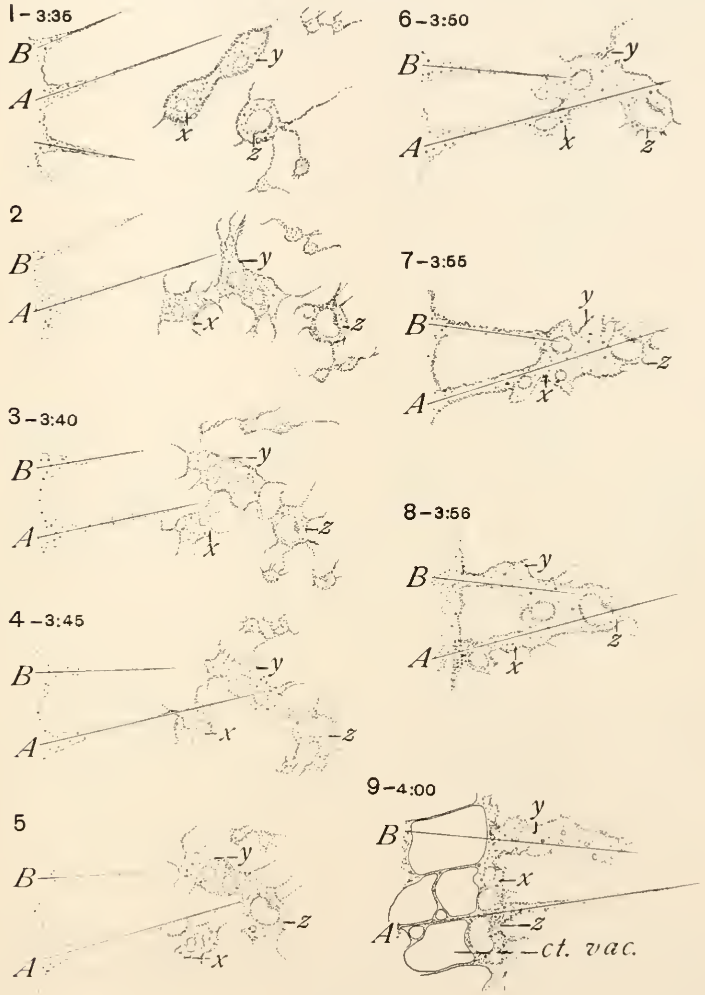
The first step after severance is usually the rounding up of the broad basal end of the axopod as an irregular granular mass, into which the extended portion is gradually withdrawn (Record 4; Figs. 1, 2, 3), and from which new pseudopodial extensions of great length are thrown out (Fig. 4). During its migration, this body becomes increasingly vacuolated (Record 1, Figs. 10–18). No axial cores were ever observed in its pseudopodia (filopodia).



Record 1, Figs. 1–18. Changes of form exhibited by a severed axopod before reincorporation. Time lapse, 2:00–2:40. *P*, pseudopodial extensions. *V*, newly formed vacuoles. *U. A.*, axopod still unsevered from the main body.

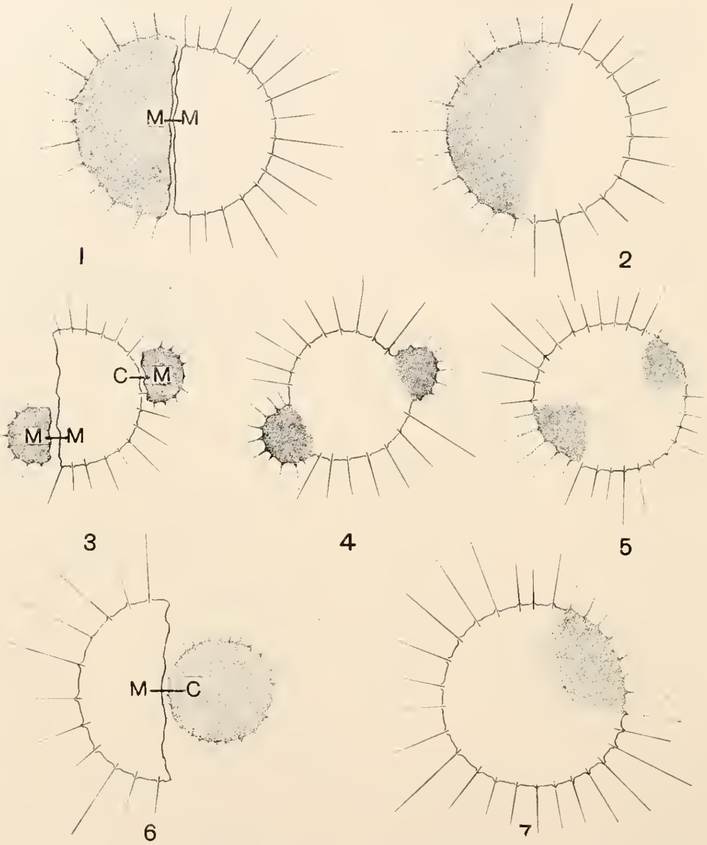
A number of severed axopodia may unite to form a loose network (Record 2, Figs. 1 to 9), which behaves as a single unit.

The first sign of protoplasmic union may occur at either the tip or the side of an axopod. Fragments reincorporated near the tip, increase the size of an axopod at that point to several times the original diameter, and have the appearance of a large lateral


1–3:36
B
A
y
x
z
6–3:50
B
y
A
x
z
2
B
A
y
x
z
7–3:55
y
B
z
A
x
3–3:40
B
y
A
z
x
8–3:56
y
B
z
A
x
4–3:45
B
y
A
x
z
9–4:00
y
B
x
A
z
ct. vac.
5
y
B
z
A
x

Record 2. Figs. 1–9. Protoplasmic network formed from a number of severed axopodia. *x*, *y*, *z*, individual axopodia, gradually fusing, and finally reincorporated as a single mass. Time lapse, 3:35 to 4:00. *A* and *B*, the two unsevered axopodia chiefly concerned in the reincorporation process. *Ct. vac.*, vacuoles of the cortical layer of the main body.

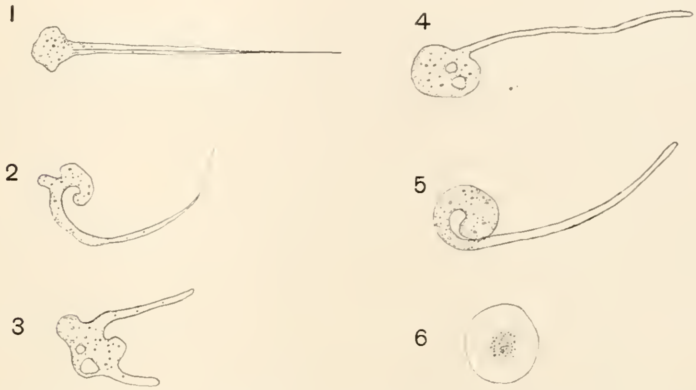
swelling (Record 5, Fig. 6). This mass travels centripetally, flattening as it goes, and fuses completely at the level of the cortical surface (Record 5, Fig. 7; Record 1, Fig. 18).



Record 3, Figs. 1–7. Diagrammatic representations of different types of grafting between stained and unstained vegetative masses of *Actinosphærium eichhornii*. Stippled areas represent portions of animals stained with dilute neutral red. Clear areas represent portions of unstained animals (white). *M–M*, medulla grafted to medulla. *C–M*, cortex grafted to medulla.

Axopodia cut from one animal and carried on the needle point to the immediate region of another, will be incorporated if the protoplasm is still capable of locomotion. Dead (motionless) axopodial masses are refused.

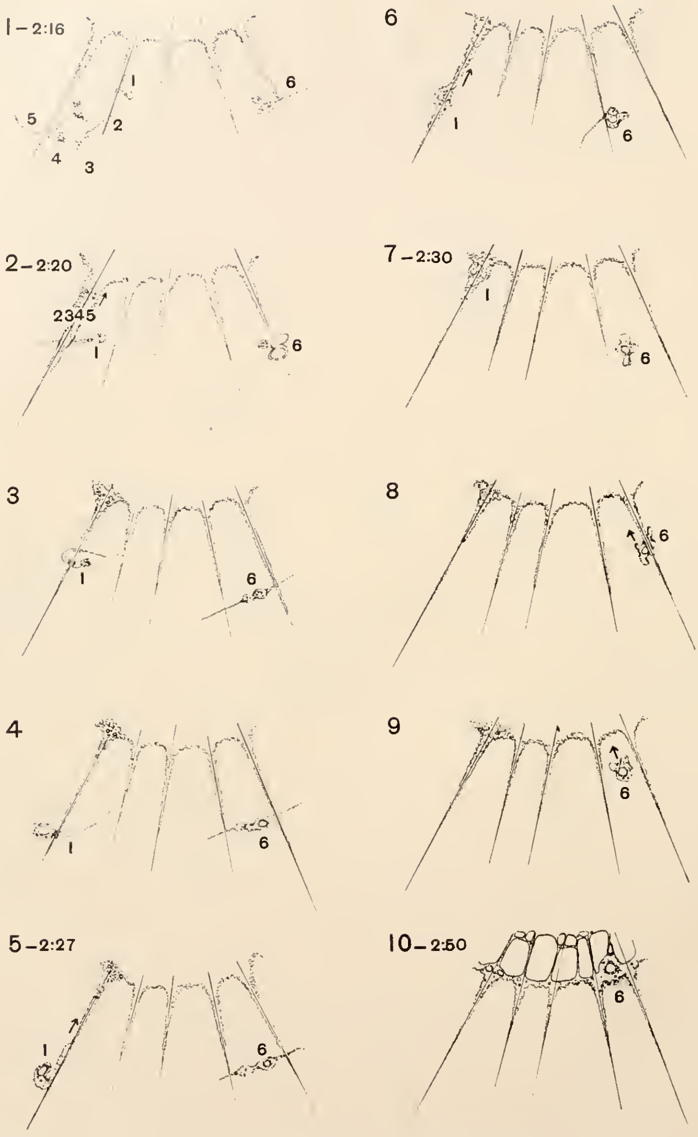
Frequently, severed axopodia were observed which approached very closely to an animal without fusing, and subsequently moved away. If fusion had not occurred at the end of 40 to 50 minutes, the pieces became spherical and quiescent, and showed signs of hyalinization and disintegration (Record 4, Fig. 6).



Record 4. Figure 1 represents an axopod just severed from the main body. Figs. 2 and 3, stages during resorption of the original axopodial extension. Fig. 4, newly formed pseudopod, without axial core. Fig. 5, twisting movement which results in a clumsy locomotion. Fig. 6, hyalinization and disintegration after failure to become reincorporated.

It is demonstrated in the foregoing experiments that the highly vacuolated cytoplasm of the vegetative masses (colonial aggregates) of *Actinosphærium* is very adhesive. The ease with which artificial plastogamy or grafting is accomplished under compression, strongly suggests the possibility that the failure of Johnson, '94, to fuse vegetative individuals was due to the lack of some similar device. Also, the essential factor in the experiments of Cienkowski, '65, would seem to be the compression which he applied by the use of paper strips, rather than the approximation of two cut surfaces.

Reincorporation of severed axopodia is, in reality, the same process on an infinitely smaller scale. The fusion, in these cases, is unlike reincorporation in *Difflugia* in that it may occur at the tip or side of an unsevered axopod, or at the cortical surface of the main body.


1–2:16
1
6
5
2
4
3
6
1
6
2–2:20
2345
1
6
7–2:30
1
6
3
1
6
8
6
4
1
6
9
6
5–2:27
1
6
10–2:50
6

Record 5. Figs. 1–10. Diagrammatic representation of the process of reincorporation of six severed axopodia, numerically labelled. Time lapse for reincorporation of numbers 2, 3, 4 and 5, was four minutes; for number 1, fourteen minutes. Number 6 did not fuse with unsevered axopodia, but moved slowly toward the cortex, fusing there in thirty-four minutes.

Summary.

Grafting.

1. Merotomized portions of *Actinosphærium eichhornii* may be grafted together under slight compression.

2. Fusion occurs between two medullary, or between medullary and cortical surfaces.

3. Grafted animals regain a spherical shape, and show no sign of separation at the end of four days.

Reincorporation of Axopodia.

1. Axopodia completely severed from the protoplasmic mass may be reincorporated, either by the same or by another animal.

2. Severed axopodia often become vacuolated, and develop long pseudopodia.

3. Reincorporation may occur at either the tip or the sides of unsevered axopodia, or on the cortical surface of the main body.

4. Severed axopodia which have not been reincorporated at the end of 40 to 50 minutes, round up, become quiescent, and show signs of disintegration.

5. Motionless axopodial fragments are not incorporated.

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MOSAICISM AND MUTATION IN *HABROBRACON*.

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In a recent publication (Whiting and Whiting, 1927) the hypothesis was advanced that certain irregular types in the parasitic wasp, *Habrobracon,* were caused by incomplete maturation of the egg. Impaternate females were assumed to originate from eggs in which there was failure of second maturation division. Mosaic males from unfertilized eggs of heterozygous mothers were thought to be due to failure of extrusion of second polar body, two oötids with different genes taking part in maturation; gynandromorphs to have originated in a manner similar to that of the mosaic males, except that one oötid was fertilized. A single heterozygous mosaic female was explained by mitotic irregularity in somatogenesis.

In the present paper are presented data giving additional evidence for the hypotheses previously advanced although no more impaternate females have been found. Factors considered here are the allelomorphs black, O, light, o^l, orange, o, and ivory, o^i (eye color), (Whiting and Burton, 1926) and normal, R, and reduced, r, (wings), (Whiting, 1926). Materials involved are stock 11, (type) from Iowa City, Iowa, which when previously used in crosses with Lancaster material gave the irregular types already discussed; stock 17 (ivory) and stock 20 (ivory reduced), both of mixed origin from the Lancaster and Iowa City materials; stock 24 (type) from Lowell, Massachusetts; and orange-eyed males from various stocks.

Stock 24 has not been previously described. A female, captured September 7, 1926 in Lowell, produced 24 males and 73 females. Thirteen virgin females produced 651 males. Thirty-

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[1] The present work, begun at the University of Maine, has been carried on at the Bussey Institute under a grant from the Committee for Investigation of Problems of Sex of the National Research Council. The writer wishes to express his indebtedness to this committee for support and to the Bussey Institution for courtesy in extending space and facilities.

TABLE I.

PROGENY FROM Oo^iRr FEMALES, VIRGIN OR CROSSED WITH VARIOUS MALES.

Parents.			Regular Offspring.								Freaks.		
F_1 ♀♀ Oo^iRr.		♂♂.	♂♂.				♀♀.				Biparental ♂♂.	Mosaic and Mutant ♂♂.	⚥⚥.
No.	Source ♀ ♂		Type.	Ivory.	Reduced.	Ivory Reduced.	Type.	Orange.	Reduced.	Orange Reduced.			
24	20 × 11		994	989	940	934						283, 289, 319	
14	11 × 20		468	494	417	449						286, 287	
9	11 × 20	Orange	64	73	62	54	322	296			292	305	303
7	11 × 20	Orange Reduced	81	76	60	60	141	98	114	128	290, 291, 294		296, 302
6 6	20 × 24 24 × 20		308	315	277	298							
25 12	20 × 24 24 × 20	Orange	430	465	414	415	1,207	1,201			293?, 297, 298	306	
3 6	20 × 24 24 × 20	Orange Reduced	126	123	107	98	96	104	55	70	293*a*?, 295		
3	20 × 11	Ivory Reduced	55	52	36	46	100	Ivory 109	94	Ivory 89			
Total regular showing segregation.			2,526	2,587	2,313	2,354	337	311	263	287			

six mated females produced 1417 males and 1129 females. These wasps were of normal appearance, like Lancaster "type" stock 1, but mesosternum was very "sooty" and 9 males and 6 females had breaks in wing vein r_4.

Reciprocal crosses were made between stocks 20 and 11 and between stocks 20 and 24. The F_1 females were in some cases isolated as virgin, in others bred to orange males (stocks 3, 12, 13) or orange reduced males (stock 19). Results in the segregating generation (from females Oo^iRr virgin or mated to oR or to or males) are given in Table 1 in which are also included three fraternities from F_1 females which had mated to their ivory reduced, o^ir, brothers.

Disregarding for the moment the 21 freaks which are designated by numbers at the right of the table, it may be seen that offspring fall into the expected classes on basis of independent segregation of ivory and reduced and dominance of orange over ivory. Males segregate in all cases while females segregate only when their fathers are recessive. None of the regular males are orange while ivory is concealed in all females except those with ivory fathers. Females segregating into the four classes are summarized as are all regular males at bottom of table. Reduced fall below expectation both in males, 47.7 per cent., and in females, 45.9 per cent. Recombinations have taken place in 50.1 per cent. of males and in 47.9 per cent. of females segregating for both factors. There is thus no evidence of linkage. In order to determine whether there might be linkage at some period during the mother's life or some effect of age of mother upon recombination of factors, segregating progenies of all cultures were added according to vials through which mothers were passed. No significant difference from average was found.

The infrequent types, "freaks," may now be considered.

Biparental Males.

Biparental males, formerly called anomalous or, because they are to be distinguished from other males by possessing paternal traits, patroclinous males, resemble their sisters in showing dominant characters inherited from either or both parents (Whiting, Anna R., in press). They are almost or quite sterile. Their few daughters tend toward morphological abnormality and are usually sterile.

The nine males classed as biparentals in Table I are distinguished from the regular males by possessing orange eyes of paternal origin. They occur only in bisexual fraternities and only from vials containing females, in other words, before their mother's supply of sperm has become exhausted. Freaks 293 and 293*a* were not tested but are probably biparental. The remaining seven were almost or quite sterile. The four with long-winged father had long wings as expected. Of the five with reduced father, only one (290) had long wings received from its Rr mother.

Mutant and Mosaic Males.

Mutant Male 289.—An F_1 female from ivory reduced female by type male produced in addition to males,—type 75, ivory 61, reduced 69 and ivory reduced 60, an orange reduced male (289) (in vial *f*) with compound eyes rather dark and ocelli of typical orange color. He was mated with eight different females over a period of forty-four days producing daughters by each of them. A subsequent mating after ten days resulted in males only (58) indicating sterility due to age. Of the eight females producing daughters, two, orange reduced, produced 60 orange reduced daughters; five, ivory reduced, produced 172 orange reduced daughters, and one, type carrying reduced, produced 13 type and 4 reduced daughters. The eight mothers also produced 160 normal matroclinous males. Nine of the orange reduced daughters with orange reduced mothers were tested and produced only orange reduced offspring. Freak 289 therefore bred as he appeared somatically transmitting reduced in at least 236 cases and non-black (orange or ivory) in at least 232 cases of which at least 181 were certainly orange.

Mutant Male 319.—An F_1 female from ivory reduced female by type male produced in addition to males,—type 60, ivory 75, reduced 64 and ivory reduced 92, an orange reduced male (319) (in vial *g*) with compound eyes rather dark and ocelli typical for orange. He was mated over a period of fourteen days to eleven ivory reduced females. There were produced 490 ivory reduced males, 2 orange reduced (biparental) males, and 640 orange reduced females. Two subsequent matings resulted in males only, 207. Nine orange reduced daughters produced 218 orange reduced males and 210 ivory reduced males. No new types appeared.

Mutant Male 308.—Freak 305 (see below) had eyes which appeared mosaic for black and orange, gonads mosaic for black and ivory. All of his offspring had long wings. Four of his type (di-heterozygous Oo^iRr) daughters from an ivory reduced mother (two had mated to ivory reduced brothers) produced males—type 129, ivory 139, reduced 134, and ivory reduced 121 and females—type 37, ivory 29, reduced 20, and ivory reduced 19. In one of these four fraternities which included females in vials *a, b,* and *c,* but males only in vial *d* there occurred, in vial *d,* an orange (deep red) long male (Freak 308).

Freak 308 was tested by mating to twelve ivory reduced females over a period of fifteen days. There were produced males—ivory reduced 701, orange long (biparental) 13, and females orange long 244. Two of these orange biparental males were tested by mating, twice each at an interval of eight days. Males of maternal type only appeared, 67 and 104 respectively, showing that biparental males from Freak 308 tend like other biparental males to be sterile.

Seven orange virgin daughters of Freak 308 segregated males—orange, ivory, orange reduced and ivory reduced as expected. Selection of two deep red of these "orange" males and crossing with ivory reduced females gave orange males in F_2 that were of the usual variability. No effect of selection immediately following mutation appeared.

Freaks 289, 319, and 308 may be regarded as mutants to orange among the expected four classes of males from Oo^iRr mothers.

Mosaic Male 286.—An F_1 female virgin from type female by ivory reduced male produced in addition to males—type 40, ivory 48, reduced 35, and ivory reduced 31, a black-eyed male with left wing reduced, right long (Freak 286), occurring in vial *d* (Fig. 1). He was set with seven different females over a period of eight days. He made prolonged and vigorous attempts to mate which appeared at times successful. No daughters were produced among 427 sons. Examination showed external genitalia to be abnormal in the presence of an extra right external clasper, as also a curious chitinous structure suggesting an extra malformed penis (Fig. 2). Sterility of this male may be attributed to his inability to complete copulation. His potential breeding capacity is therefore unknown. It is postulated that the first oöcyte division was equational for Rr and that two oötids containing R and r respectively took part in cleavage.

Mosaic Male 287.—An F_1 female virgin from type female by ivory reduced male produced in addition to males—type 37, ivory 38, reduced 36, and ivory reduced 42, an ivory-eyed male (Freak 287) with right wings reduced, left long, occurring in vial d (Fig. 10). Eyes and ocelli were typical for ivory.

Freak 287 was mated to each of thirteen females over a period of fifty-two days. Daughters were produced by all of them. Three subsequent matings resulted in males only, 276, indicating sterility due to age. In addition to the 270 males of maternal type produced by the first thirteen females there were the following biparental offspring:

Six ivory reduced females produced 113 black reduced, 8 males and 105 females.

Three orange/ivory reduced females produced 120 black reduced, 5 males and 115 females, and 1 ivory long female.

Four homozygous orange reduced females produced 26 black reduced, 4 males and 22 females.

One homozygous ivory long female produced 24 type, 2 males and 22 females.

Freak 287 therefore had the capacity to produce biparental males, 19 to 265 females. Biparental males from recessive mothers indicate as do females what genetic factors are in the sperm. Black biparental offspring number 284 of which those with reduced mothers, 260, were all reduced. The single ivory long daughter from orange/ivory reduced mother represents the combination visible in the eyes and left wing of Freak 287.

Each of two of the reduced biparental males with ivory reduced mother was mated once. Ivory reduced males only resulted, indicating that biparental sons of mosaic males tend, like other biparental males, to be sterile.

The origin of Freak 287 may be expressed

First polar body	$\mathrm{Oo^iRr}$
Cleavage nuclei	$Or\ o^iR$

Italics are used to designate origin of gonads.

Mosaic Male 307.—Five type females, F_1 from ivory female (stock 17) by type male (stock 24) produced males only—type 412 and ivory 408 and in vial h a male (Freak 307) with pale orange eyes and ocelli typical for orange. He was mated with fourteen ivory females over a period of twenty-five days. Besides the 939 ivory sons there were 470 biparentals, all black, 9 males and 461 females.

Two of these biparental males were tested by mating, each to two females at an interval of eight days. Males only resulted, 66 and 104 respectively. Biparental males from Freak 307 are therefore of the usual sterile type.

Three daughters of Freak 307, produced males only, type 139, ivory 154.

Reduced is not concerned in the production of Freak 307. His origin may be expressed

First polar body	$\mathrm{Oo^i}$
Cleavage nuclei	$O\ \mathrm{o^i}$

If it be assumed that $\mathrm{o^i}$ mutated to o, Freak 307 is not only a mosaic but also a mutant. This question is discussed below.

Mosaic Male 321.—A black reduced male was mated to an ivory reduced female. 2 ivory reduced males and 28 black reduced females resulted. One of these daughters produced ivory reduced males 2, and a reduced male (321) with ocelli and left eye black, right eye black with red or orange area ventrally (Fig. 9). Long wings are not involved in this experiment. Freak 321 was mated to twenty-five ivory females over a period of twelve days. Ivory males 1816, black (biparental) males 66, and black females 806, resulted.

Freak 321 may be regarded either as a mosaic of black and ivory in which the ivory has taken on a reddish appearance due to proximity of "black" tissue or as a simultaneous mosaic and mutant. Its origin may be from a binucleate egg, or from a normal egg with subsequent mutation of O to o.

Mosaic Male 323.—A type male was mated with an ivory reduced female. Type females only (26) resulted. One of these produced 44 males—type, ivory, reduced, ivory reduced as expected and in vial *d* a male, Freak 323, with long wings and mosaic eyes and ocelli (Figs. 5 and 6). He was mated with sixteen ivory reduced females over a period of eight days. Ivory reduced males 158, type (biparental) males 16, and type females 280, resulted.

Freak 323 is comparable with Freak 321 in that light areas of eyes are orange rather than ivory. They are, however, much more extensive. His origin on basis of two oötids may be expressed:

$$\frac{\text{First polar body}\qquad Oo^irr}{\text{Cleavage nuclei}\qquad OR \mid o^iR}$$

o^i may have mutated to o or the orange color may be purely somatic.

Mosaic Male 283.—An F_1 female from ivory reduced female by type male produced in addition to type males 50, ivory males 36, reduced males 38, and ivory reduced males 41, an orange-eyed male (Freak 283) with long wings occurring in vial *c*.

Two matings of Freak 283 with ivory long females (stock 17) resulted in 133 ivory males and 161 type females. The production of black-eyed daughters indicates that Freak 283 was a mosaic involving black. Fifteen of these type daughters isolated as virgin produced males—type 321, ivory 277, reduced 252, and ivory reduced 278. The production of reduced males proves that Freak 283 transmitted reduced and was therefore mosaic of long and reduced, as well as of black and ivory (or orange).

A mating of Freak 283 with orange reduced (stock 19) female resulted in orange reduced males 41, reduced females 56, showing directly that Freak 283 produced reduced only. Five of these reduced females produced reduced males 238, and orange reduced males 208, as expected. Freak 283 was also mated to type female (stock 1). This resulted in type males 93, and type females 30. Six of these females produced males—type 99 and reduced 83, and one which was mated to Freak 283 produced females—type 14, and reduced 15.

Freak 283 was also mated with three ivory reduced females. There resulted ivory reduced males 28, and reduced females 139, as well as a gynandromorph (Freak 304) with reduced wings and eyes mosaic of black and orange discussed below. Eight of these females which had mated with their ivory reduced brothers produced males—reduced 30, ivory reduced 34, and females—reduced 93, and ivory reduced 92, as expected.

Summarizing the results of the eight matings of Freak 283 extending over a period of fourteen days, we find that he transmitted black, O, in at least 363 cases and reduced, r, in at least 232 cases. In other words he bred as a black reduced and no new factors or unusual types appeared except the gynandromorph, 304, discussed below. His origin on the basis of egg binuclearity may be expressed.

First polar body	$\mathrm{Oo^iRr}$
Cleavage nuclei	$Or \mid \mathrm{o^iR}$

Freaks 283 and 307 differ from freaks 321 and 323, in that there is no trace of black in the eyes. Orange appearance is therefore not due to proximity of black facets, but to some physiological influence from "black" tissue or to mutation.

Mosaic Male 306.—An F_1 female from type female by ivory reduced male was mated with an orange long male (stock 12). From vials *a–d* there appeared males—type 9, ivory 13, reduced 6, ivory reduced 9, and females—type 26, and orange 27, as expected. Subsequently (vials *e–h*) there appeared males—type 26, ivory 26, reduced 34, ivory reduced 17, and a long-winged male with light orange eyes and mosaic ocelli appearing in vial *h* (Freak 306, Figs. 7 and 8). As may be seen from the figure the right ocellus is without pigment, even the slight amount characteristic of "orange" being absent. Anterior and possibly also left ocelli are mosaic. That the thorax is also possibly mosaic is indicated by its lighter color on right side.

Freak 306 was mated over a period of thirty-eight days with nine ivory reduced females and with five ivory long carrying reduced. In addition to the 814 males of maternal type there were 601 ivory long daughters. 464 of these (from ivory reduced mothers) prove that Freak 306 transmitted long. Of the remainder from Rr mothers, about half must have been long because of factor R in the sperm.

Freak 306 therefore breeds like an ivory long. The ocelli certainly contain black and either ivory or orange. The compound eyes are of definite orange appearance. Origin may be expressed

$$\frac{\text{First polar body}\qquad \mathrm{Oo^{i}rr}}{\text{Cleavage nuclei}\qquad \mathrm{OR} \mid o^{i}R}$$

"Black" tissue is regarded as present because of the ocelli. If orange appearance be due to mutation of o^{i} to o then there are three types of tissue here, O, o, and o^{i}.

Mosaic Male 305.—An F$_1$ female from type female by ivory reduced male was mated with an orange long male (stock 3). There were produced males—type 23, ivory 24, reduced 18, and ivory reduced 28, and females—type 94, and orange 74 as expected. There also appeared in vial *g* along with type and orange sisters, a male, Freak 305, with long wings, orange ocelli, and eyes mosaic of black and orange (Figs. 11 and 12).

Freak 305 was mated with eleven females over a period of twenty-nine days. Three subsequent matings resulted in males only, 297, indicating sterility due to age. The eleven females producing daughters gave in addition to 529 matroclinous sons, the following biparentals:

Three homozygous ivory females produced type females 6, ivory females, 122.

Eight ivory reduced females produced type females 19, ivory long male 1, ivory long females 243, ivory females with wings unexpanded 8.

Freak 305 therefore transmits long wings, 263 cases, both in association with black, 19 cases, and ivory, 244 cases.

Table 2 shows distribution of the 25 black and 374 ivory progeny according to age of Freak 305.

Origin of Freak 305 may be expressed

$$\frac{\text{First polar body}\qquad \mathrm{Oo^{i}rr}}{\text{Cleavage nuclei}\qquad OR \mid o^{i}R}$$

Gonads are evidently mosaic for O and o^{i}, eyes for O and o^{i} or O and o.

DIPLOID MOSAICS.

Mosaic Female 313.—An orange long female (stock 10) mated with an ivory reduced male (stock 20) produced orange long—

males 20 and females 6 and an orange female (Freak 313) with left wing reduced, right long (Figs. 22 and 23) and fifteen joints in each antenna, typical for female.

TABLE II.

DISTRIBUTION OF BLACK AND IVORY PROGENY ACCORDING TO AGE OF MOSAIC MALE 305.

Progeny.	Age in Days since Eclosion.											Total.
	4	5	7	12	13	16	18	26	28	30	32	
Black.........		4		1	5	2	2	1	10			25
Ivory.........	9	56	10	49	64	48	39	23	74	1	1	374

Freak 313 which had mated with her orange brothers produced males—orange 13, ivory 9, orange reduced 13, ivory reduced 10, and females—orange 39. Two of these females, when tested, produced reduced sons while five gave only long. Eggs of Freak 313 bearing R therefore numbered 27 while eggs bearing r numbered 25.

Freak 313 may be regarded as a heterozygous female, oo^iRr, in which some somatic mitotic irregularity occurred eliminating R in the development of the left wing.

It may be noted that this mosaic female, unlike the mosaic males thus far discussed, had a homozygous mother.

Mosaic Biparental Male 318.—An ivory reduced female mated with an ivory long male produced ivory reduced males, ivory long (biparental) males, and ivory long females and ivory male (Freak 318) with left wing reduced, right wing long and terminal joints of right antenna fused (Figs. 3 and 4).

Freak 318 differs from mosaic males previously described in having a homozygous mother. It is evidently biparental, receiving R from its father. Its mosaicism was regarded as comparable in origin to that of female, 313 (somatic mitotic irregularity eliminating R). Being biparental, it was expected to be sterile or to produce a few sterile daughters. It was mated with six females over a period of twelve days. Nothing but males of maternal type appeared, totalling 946.

GYNANDROMORPHS.

Gynandromorph 296.—An F_1female from type female by ivory reduced male was mated with an orange reduced male (stock 19). There were produced males—type 23, ivory 29, reduced 28, ivory reduced 18, and females—type 34, orange 25, reduced 26, orange reduced 35, and in vial *c* a gynandromorph (Freak 296) with ivory eyes, right wing long, left reduced, sixteen joints in right antenna, 24 in left, and female abdomen (Figs. 18, 21). Measurements showed ocelli of male type. Responses resembled those of the male.

Since the father was orange the male parts (head with ivory ocelli and compound eyes) were of maternal origin. Difference in primaries indicates that one oötid contained R, the other r.

Gynandromorph 302.—An F_1 female from type female by ivory reduced male was mated to an orange reduced male (stock 19). There were produced males—type 14, ivory 17, reduced 10, ivory reduced 11, and females—type 39, orange 32, reduced 33, orange reduced 34, and in vial *f* a gynandromorph (Freak 302) with black eyes and ocelli, male head (right antenna with 20 joints, left with 21 and ocelli of male size), long wings and female abdomen. Responses were characteristic of the male.

Head and wings show maternal traits. Antennæ and ocelli and therefore presumably compound eyes are male. Whether wings are male like head (haploid) or female like abdomen (diploid) is uncertain.

Gynandromorph 303.—An F_1 female from type female by ivory reduced male was mated to an orange long male (stock 12). There were produced males—type 10, ivory 14, reduced 11, and ivory reduced 8 and females—type 36, and orange 46, and a gynandromorph (Freak 303) in vial *b* with ivory eyes and ocelli, long wings, male head, antennæ with 23 joints) and female abdomen (Fig. 24). Responses were characteristic of the male.

Eye color indicates maternal origin of male parts (head). Wing character may have been derived from either parent and may be either haploid (male) or diploid (female).

Gynandromorph 288.—An ivory reduced female mated to a light (black eyes, dilute ocelli), o^1, long male produced 4 ivory reduced males, 3 light long males, (biparentals), 23 light long females and a gynandromorph (Freak 288) in vial *c* with ivory eyes, male head (23 joints in each antenna), left wing long, right reduced, and abdomen female (Figs. 19 and 20). Pupal membrane adherred to tip of abdomen and specimen was nearly dead when found, perhaps diseased. Darker color of right side of sternum indicated that right side of thorax, with wings reduced (matroclinous) was probably male. This is consistent with fact that head, also matroclinous, is male.

Gynandromorph 322.—An ivory reduced female mated to a black reduced male produced 10 ivory reduced males, 46 black reduced females and in vial *b* an ivory reduced gynandromorph (Freak 322) with male head (20 joints in each antenna) and a female abdomen. Reactions were male but weak. Long wings are not concerned in the cross. Eye color shows male parts to be maternal in origin.

Gynandromorph 304.—An ivory reduced female mated to Freak 283, orange long male breeding as black reduced (see above) produced ivory reduced males 12, black reduced females 71, and in vial *b* a gynandromorph (Freak 304) with reduced wings and eyes mosaic for black and orange (Figs. 13–17). Left antenna is clearly female (15 joints), right is male but with terminal joints fused. Ocelli are mosaic, the right and anterior with considerable black pigment, predominantly female; the left with very little pigment clearly larger than the right and exclusively or predominantly male. Compound eyes are mosaic for black and orange. Abdomen is entirely female, sting split, and right gonapophysis, in agreement with right antenna, deficient. The left secondary is considerably longer than the right indicating femaleness.

Responses proved to be exclusively male. Repeated and vigorous attempts were made to mate with females.

Freak 304 is evidently an intricate mosaic of male and female parts, very different in this respect from any gynandromorphs previously found in this species.

The size of the left ocellus indicates that it is male and its reduced amount of pigment shows its maternal origin. Black regions of eyes indicate paternal influence and are assumed to be female (diploid). Orange regions of eyes are presumably male (haploid) and of maternal origin, ivory being changed to orange at least in appearance.

Gynandromorph 325.—An ivory reduced female mated to a black reduced male produced 7 black reduced females and in vial *a* a small-sized gynandromorph (Freak 322) with male head and female abdomen. Eyes and ocelli were entirely black, the latter of male size. Wings were wrinkled with skin adhering. Abdomen was collapsed, genitalia immature. Sternites were of female type. Antennæ were deficient. The left had seventeen joints, the right eighteen with the two terminal fused. These antennæ are interpreted as male since counts of joints of over eighteen hundred female antennæ previously made showed none above sixteen and this number occurred in the larger individuals only.

The specimen was weak when found. Reactions could not be tested.

Freak 322 is the first gynandromorph found with male parts patroclinous. Male biparentalism is indicated by the general abnormality and weakness of the specimen. As explanation for origin, either egg binuclearity and dispermy or somatic mitotic irregularity may be suggested.

Frequency of Occurrence of Mutants and Mosaics.

Of the 14,023 wasps recorded in Table I., 9,787 (normal males 9,780, mutant males 3, and mosaic males 4) may be regarded as coming from unfertilized eggs and 4,236 (normal females 4,224, biparental males 9, and gynandromorphs 3) from fertilized eggs laid by diheterozygous mothers, Oo^{i}Rr. These mothers came in all cases from crosses of stock 20, ivory reduced, and a type stock, either 11 or 24.

Mosaic and mutant males and gynandromorphs number 9 among the 7,908 descended from stock 11, while there is but one mosaic male among the 6,115 descendents of stock 24. This may indicate a difference in hereditary tendency toward binuclearity in favor of stock 11.

Among the eleven haploid freak males above discussed, three (289, 308, 319) were mutants but not obviously mosaics, two (286, 287) were mosaics but not obviously mutants, and six (283, 305, 306, 307, 321, 323) were mosaics and at least apparently mutants for they showed orange color in eyes. Since two (283, 307) at least of the latter would not have been tested and detected as mosaics had they not been orange, it was thought that other males apparently segregating in normal manner might be mosaic also. Gonads might differ from soma.

Accordingly F_2 males of normal appearance from a cross of ivory reduced (20) by type (11) were tested by mating to ivory reduced females. Thirty-five type bred as type (offspring totaling ivory reduced males 172, type males 24, type females 750), forty-two ivory bred as ivory (offspring totalling ivory reduced males 218, ivory males 28, ivory females 911), thirty-eight reduced bred as reduced (offspring totalling ivory reduced males 254, reduced males 21, reduced females 1,217) and twenty-nine ivory reduced bred as ivory reduced (offspring totalling 77 males and 285 females. Thus of 144 normally segregating males from Oo^iRr females none showed gonads different from soma.

Hypotheses Suggested.

The data above presented show that orange eye color may appear in males from eggs laid by mothers heterozygous for black and ivory, Oo^i, in at least two or possibly three different ways. Biparental males obviously receive the factor O from their father. Males with eyes mosaic for black and orange have thus far bred as black (321, 323), as ivory (306), or as black and ivory mosaic (305). In the case of three of these it may be supposed that proximity of black facets may cause the " ivory " to take on an orange appearance, but number 306 had compound eyes entirely orange with dark pigment appearing in the ocelli only. Moreover since two orange males (283, 470), breeding as black showed no trace of black pigment, it must be supposed that influence of " black " tissue in changing ivory to orange is not merely an optical effect from proximity of black facets, but is due to some physiological (enzymatic?) influence which actually transforms ivory to orange. If this be the case, it must be supposed that in number 287 (ivory breeding as black-ivory mosaic) the " black " tissue did not involve the " enzyme-producing gland."

An alternative to this " somatic " explanation is to suppose that actual genetic change has taken place. In the mosaic males gonads have not included this orange tissue. It may be suggested that the three orange mutants (289, 308, 319), although not obviously mosaics, may have been derived from binucleate eggs carrying O and o^i for their mothers were Oo^i.

The hypothesis of mutation of o^i to o caused by association with O tissue brings the various phenomena into harmony. Evidence both that it is o^i rather than O which has changed and that this change has taken place in somatogenesis rather than in ovogenesis is afforded by the mosaic-eyed (black-orange) gynandromorph 304 in which O must have been derived from the male parent for the mother was o^io^i.

Evidence decisive between the somatic and the genetic hypotheses has not yet appeared. A mosaic male from Oo^i female producing o gametes or a male with eyes mosaic of orange and ivory would be of much interest in this connection.

Summary.

1. Independent segregation of ivory (eye color) and reduced (wings) is shown in progeny of Oo^iRr females.

2. When such females are mated to orange, o, males there occur among the normal offspring, orange (biparental) males with characteristic sterility.

3. One female from RR female by r male and one sterile biparental male from rr female by R male were each characterized by possessing one long and one reduced wing. Elimination of chromosome bearing R is suggested as explanation.

4. Eight mosaic males were produced by Oo^i females. Of these—one was sterile, four produced black daughters only, one ivory only, and two both black and ivory. It is suggested that these males arise from binucleate eggs and that gynandromorphs are produced when one nucleus of such eggs is fertilized.

5. Of seven gynandromorphs six showed male parts matroclinous while in one they appeared patroclinous. In the latter case they may have been diploid, comparable to ordinary male biparentalism.

6. Two mosaic males had eyes completely orange while four mosaic males and one gynandromorph had eyes mosaic of black and orange. Since presumably only black or ivory entered into

the gametes producing these mosaics, the orange color must have been due to some somatic physiological effect or to mutation.

7. Three mutant orange males showing no mosaicism and breeding as orange were produced by Oo^iRr females.

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EXPLANATION OF PLATE I.

FIGS. 1–24. Camera lucida drawings of various parts of mosaics of *Habrobracon juglandis.*

Figs. 1, 4, 10, 13, 18, 19, 22. × 10.

Figs. 3, 11, 12, 14, 15, 17, 20, 21, 23, 24. × 22.

Figs. 2, 6, 9. × 30.

Figs. 5, 7, 8, 16. × 63.

Figs. 1–12 are from mosaic males.

Figs. 13–21 and 24 are from gynandromorphs.

Figs. 22 and 23 are from a mosaic female.

Figs. 1 and 2, wings and ventral view of genitalia of mosaic male 286.

Figs. 3 and 4, right antennæ and wings of mosaic male 318.

Figs. 5 and 6, dorsal view of ocelli and anterior view of head of mosaic-eyed male 323.

Figs. 7 and 8, dorsal and anterior view of ocelli of mosaic male 306.

Fig. 9, right view of head of mosaic-eyed male 321.

Fig. 10, wings of mosaic male 287.

Figs. 11 and 12, dorsal and ventral views of head of mosaic-eyed male 305.

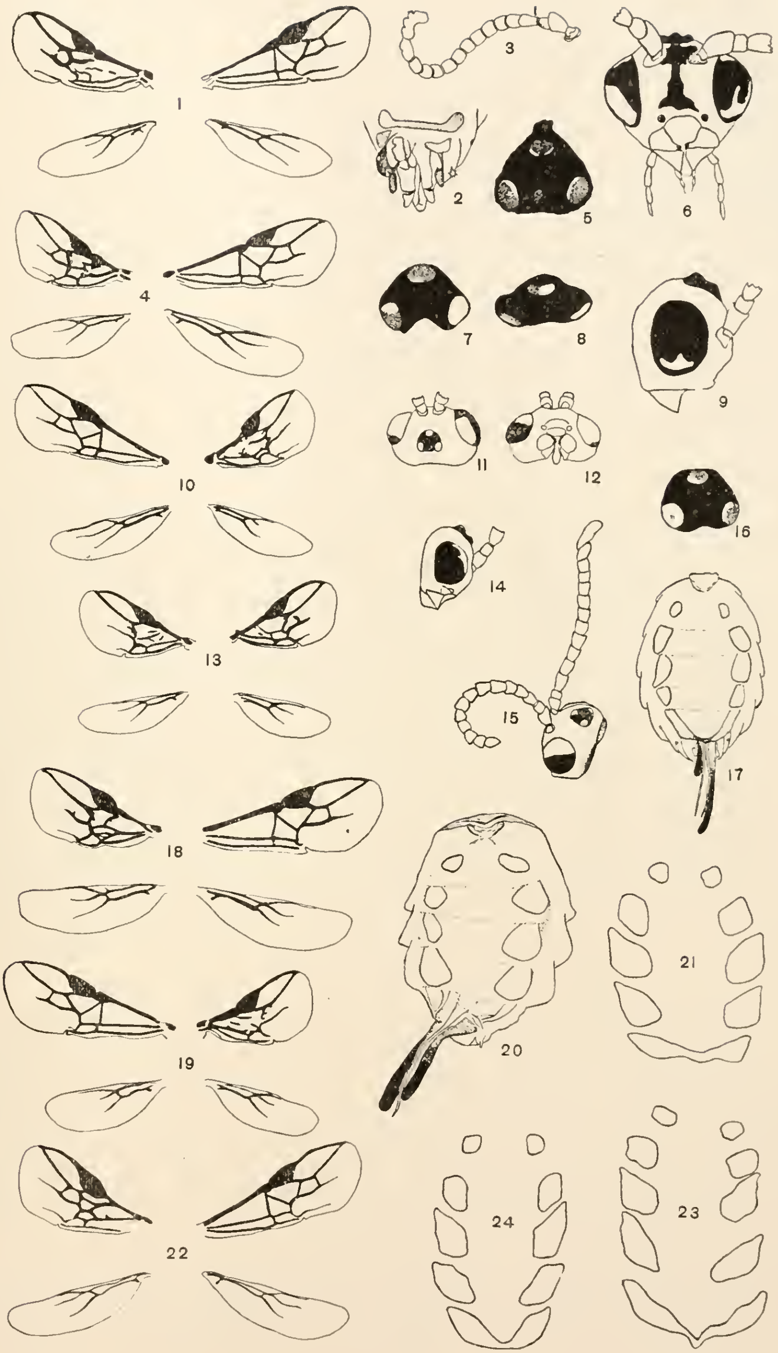
Fig. 13, wings, Figs. 14 and 15, right and dorso-sinistral views of head, Fig. 16, dorsal view of ocelli and Fig. 17, ventral view of abdomen of mosaic-eyed gynandromorph 304.

Figs. 18 and 21, wings and abdominal sternites of gynandromorph 296.

Figs. 19 and 20, wings and ventral view of abdomen of gynandromorph 288.

Figs. 22 and 23, wings and abdominal sternites of mosaic female 313.

Fig. 24, abdominal sternites of gynandromorph 303.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P. W. WHITING.

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OVULATION IN THE FOUR-TOED SALAMANDER, *HEMIDACTYLIUM SCUTATUM*, AND THE EXTERNAL FEATURES OF CLEAVAGE AND GASTRULATION.

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Introduction.

Incidental to the collection of material for a study on the germ cells of *Hemidactylium scutatum* the writer brought to the laboratory a female of this species which subsequently deposited a number of ova while under observation. The eggs laid proving fertile, the progress of cleavage and gastrulation was carefully noted. Though the observations made cover but a very limited material, they serve to supplement the work of Bishop ('18) who begins his account of the development of Hemidactylium with the neural plate stage; moreover, ovulation in this species seems not to have been previously observed and reported. For these reasons the observations made are here reported, even though the limited material prevents consideration of but little more than the superficial features of cleavage and gastrulation.

Ovulation in *Hemidactylium*.

The female on which these observations were made was captured in the sphagnum bog on the shores of Mud Pond near Ithaca,[1] the site of Bishop's discovery of the egg-laying habits of this species some years before. This particlar female was found in a sphagnum hillock together with two eggs, on the morning of May 11, 1923. She was brought to the laboratory the afternoon of that day, and placed in a small glass dish of sphagnum, together with the eggs assumed to be hers. About 9 P.M. of the same day it was noted that eight eggs were present, all but three of which were scattered over the bottom of the dish. The female had been partially concealed by the sphagnum, and was of course necessarily disturbed by the attempt to locate her and her eggs for observation. It would seem, however, that her position when first accurately observed was probably but little changed, since she was in contact with two ova which later observations would indicate had been but recently extruded. She lay with her back turned against the bottom of the dish, her abdomen upturned against the overlying sphagnum. Two eggs lay above her, still in contact with her ventral body wall immediately cephalad of the cloacal opening, but adherent to the overlying sphagnum; by gently pulling upon this sphagnum the eggs could readily be pulled away from the body of the female, who was not at all disturbed by this procedure, but remained quietly in position. Her body was somewhat curved, so that the middle part of her abdomen was in contact with the side of the dish; the end of her tail also lay in contact with the side of the dish, elevated along it and not in contact with the bottom.

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[1] This study was carried on in the laboratories of the Department of Histology and Embryology of Cornell University. The writer wishes to acknowledge the kindness of Dr. B. F. Kingsbury at whose suggestion the observations herein reported were undertaken.

With the body of the female in this position, it was noted at 9:14 P.M. that an egg was beginning to appear at the cloacal opening. The female made no unusual movements, but lay quietly while the egg continued to move slowly outward. In about two or three minutes it was practically expelled from the cloaca, but the female lay motionless for several minutes before a slight shift of her body left the egg attached to the overlying sphagnum.

The process of ovulation was not repeated until about 10:55 P.M. During the expulsion of this second egg the female twisted the front half of her body in such fashion that her left side was toward the bottom of the dish; the caudal part of the body however, was kept in its former position, with the dorsum against the bottom of the dish and the vent towards the overlying sphagnum. This position was retained for several minutes following the expulsion of the egg. After about twelve minutes the female was brought into a brighter light for more careful observations, and thereupon moved slowly away from the position she had maintained throughout the expulsion of the two ova. She now lay with the abdomen pressed against the side of the dish, the cephalic third of her body in a vertical position, the remainder, except for the end of the tail, extending in a horizontal direction along the junction of the side and bottom of the dish. In this position a third egg was expelled. As in previous ovulations, no unusual movements of the female were noted during the process. Preceding the appearance of the egg at the cloacal opening the female was seen to straighten her body and move the hind limbs slightly—movements perhaps necessary to facilitate the passage of the egg through the pelvis. The female then lay motionless with the abdomen pressed against the side of the dish while the egg was slowly forced from the cloacal orifice and against the glass. After the female had remained for about eight minutes with the egg in this position she was gently pushed away from the side of the dish, to which the egg remained adherent. As she showed no indication of further attempts at ovulation, the observations were discontinued. This female subsequently deposited a few other ova, but at times when she was not under observation.

The position of the female during the expulsion of the first two ova is of considerable interest, since it may possibly represent an approach to the normal. A horizontal position of the body with the ventral surface uppermost at the beginning of ovulation would prevent the eggs from dropping down into the spaces of the sphagnum, and would insure the contact and adherence of eggs successively expelled from the cloaca, since each egg as it leaves the cloacal opening would be supported upon the body of the female in contact with the egg previously extruded. With the accumulation of a number of eggs, the size of the egg mass, together with the viscid character of its surface would effectively prevent its dropping downward in the spaces of the sphagnum. Females taken in sphagnum bogs after ovulation ordinarily have their eggs loosely arranged in a mass around or over which the body of the animal is coiled.

The eggs when first expelled from the cloaca show only an extremely thin gelatinuous capsule; after several minutes this is noticeably thicker, due to the absorption of water from the moist environment. The outermost layer of this jelly is of a semi-fluid, viscid nature, which causes the eggs to adhere readily to each other or to sphagnum, glass, or other gelatinous egg envelopes, while a thin, membrane (vitelline membrane) closely surrounds the egg or embryo and separates it from the more fluid gelatinous envelope; a similar membrane of the egg of *Cryptobranchus* is stated by Smith ('26) to be identical with the zona pellucida of ovarian eggs.

Fertilization in this species is internal, as in other Urodeles. The breeding habits of the animals are quite unknown to the writer, but since males have not been found with the females near ponds at the time of ovulation (Bishop, '18; Blanchard, '23) it may be concluded that mating occurs at some previous time, and very probably in a terrestrial environment, the transfer of sperm from male to female possibly being effected by contact of the ventral surfaces of the animals. Bishop quotes C. and H. Thompson ('12) and Moesel ('18) to the effect that males and females have been taken from beneath the same log or stone during the spring months.

The female possesses the sperm-storing organ or spermatheca characteristic of Urodeles. According to Dieckmann ('27) the spermatheca of this species is unique in that it consists of but a small number of spermathecal tubules opening directly into the cloacal chamber; the latter feature is regarded as a primitive one, while the former (reduction of tubules) is characteristic of the most highly developed spermathecæ, in which the tubules open to a single duct-like tube (common tube,—a modified portion of the cloacal chamber). Dieckmann's descriptions of the cloaca and spermatheca of this species are based upon two specimens captured in August near Buffalo, New York. In one of these, spermatozoa are very abundant, in the other absent; on the assumption that *Hemidactylium* males are incapable of mating during the summer months, as are practically all Urodeles, these spermatozoa must be interpreted as having been stored for at least several weeks after a mating in the spring months. Their large number and whorl-like arrangement in the spermatheca suggest however, that no ovulation had occurred after they had been received into the spermathecal tubules.

CLEAVAGE.

The eggs laid under observation in the laboratory, together with others deposited by the same female, proved to be fertile, making possible a study of the early cleavage divisions. While the limited number of eggs necessarily prevented fixation and sectioning of successive cleavage stages, the external features of segmentation were carefully noted over a period of several hours, or until gastrulation was in progress.

In order to follow the progress of cleavage in individual eggs the ova were removed singly to numbered Syracuse watch glasses each containing a few drops of water; each egg was left in contact with a small bit of sphagnum rather than in the water. The watch glasses were transferred to the stage of a binocular microscope for observations; the position of the egg was changed as desired, either by moving the sphagnum to which it was attached, or by direct manipulation with needles. Though it was realized desirable to handle the eggs as little as possible, the difficulty of keeping them in position for viewing the vegetal hemisphere necessitated considerable manipulation. Though possibly this and other environmental factors may have contributed to certain of the atypical features of cleavage observed in some of the ova, it is nevertheless certain that eggs showing such features in some cases gave rise to normal embryos. The eggs were of course kept under observation without removal of their protective membranes.

Some of the eggs, when first viewed under the microscope, still showed the polar bodies adherent to their surfaces; these as a rule became indistinguishable after the first two or three cleavage divisions had been completed. The stage of maturation at the time of laying was not determined.

Since in none of the eggs laid in the laboratory during the night of May 11–12 did the first cleavage furrow make its appearance later than 10:30 A.M. of the following day, cleavage may be assumed to begin within from ten to fifteen hours after the egg is deposited. The egg of *Hemidactylium* apparently agrees with that of *Spelerpes* (*Eurycea*) in this respect (see Goodale, '11).

The pattern of cleavage will first be described in an egg in which the divisions proceeded in a relatively orderly, symmetrical fashion. In this egg the first cleavage furrow was first noted at

9:30 A.M.; it then extended over about a sixth of the egg circumference (see Fig. 1, *a*). From its middle point at the animal pole a short broad furrow extended at right angles to it on either side, the two furrows together forming a rather distinct cross. The short transverse furrow subsequently disappeared, being practically invisible by the time the first cleavage furrow had reached the egg equator. A similar furrow was observed in only one other egg. In another several short furrows radiating from the animal pole were formed preceding the appearance of the first cleavage furrow and disappeared after the latter had extended over a fourth of the egg circumference.

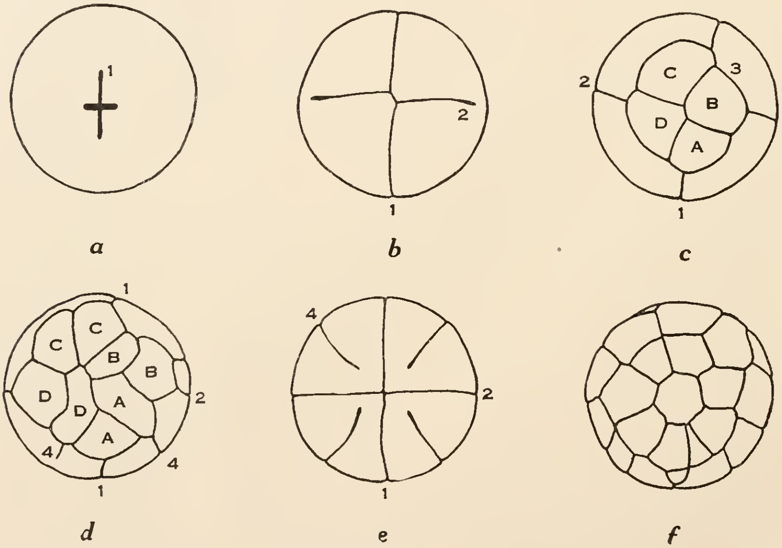

Fig. 1. Egg showing the most regular or symmetrical cleavage pattern observed. Viewed from animal pole except in *c;* cleavage furrows as numbered. *a*, 9:30 A.M. 5–12–23; *b*, 12 M.; *c*, 2 P.M.; *d*, 3:40 P.M.; *e*, vegetal hemisphere at 5 P.M.; *f*, 8:30 P.M. The origin of the micromeres in *d* is indicated by the lettering.

The progress of the first cleavage furrow through the more heavily-yolked vegetal hemisphere of the egg was at a much slower rate than its formation between animal pole and equator. From this egg and others observed we may conclude that the furrow is usually completed (on the egg surface) within about two hours after its first appearance.

The second cleavage furrows made their appearance before the first had been completed. In Fig. 1, *b* they are shown extending practically to the equator of the egg, 2½ hours later than the stage of Fig. 1, *a*. The first cleavage furrow in this egg is now complete. The second cleavage furrow apparently reaches the vegetal pole of the egg in from two to three hours.

The third set of cleavage furrows appeared about an hour after the stage of Fig. 1, *b,* and were completed within an hour, the egg at 2 P.M. having the appearance shown in Fig. 1, *c*. These furrows were roughly parallel with the egg equator and lay well up in the animal hemisphere. The four micromeres thus formed rotated a short distance in a clockwise direction to the position they occupy in Fig. 1, *c*.

The cleavage furrows of the fourth set were completed in the micromeres at 3:40 P.M., though just beginning to appear at that time in the macromeres. The direction of these furrows in *B* and *D* was roughly parallel with the first cleavage furrow; in *A* it was at right angles to this furrow, and in *C* it was meridional. With the completion of the division the daughter micromeres underwent readjustments of form and position such that the egg at 3:40 P.M. presented the appearance of Fig. 1, *d*. The fourth cleavage furrows of the macromeres are shown in this figure at an early stage of their formation; they had not been completed when the egg was sketched at 5 P.M. (Fig. 1, *e*). Their direction, while approximately meridional, was such that they would end in contact with the second cleavage furrow a short distance from the vegetal pole.

No further observations on this egg were made until 8:30 P.M., at which time it presented the appearance indicated in Fig. 1, *f*. It was impossible at this time to determine accurately the relation of any of the micromeres to those of Fig. 1, *d*.

It would appear from the above that cleavage in *Hemidactylium* is similar to that in the egg of *Eurycea* (Goodale) and is of the holoblastic unequal type characteristic of the moderately-yolked eggs of Urodeles. As in *Eurycea* and other species, however, the cleavage divisions conform to a symmetrical pattern for but few divisions if at all, and the blastomeres soon become of such irregular form and arrangement that their descent from any particular cell of the four- or eight-celled stage can not be determined.

Though irregularity of the cleavage pattern may be expected to appear during the third or fourth division in any egg of this species, as shown in Fig. 1, *d*, it is of earlier origin in a great many cases. Of ten eggs in which the furrow pattern could be determined for a stage equivalent to that of Fig. 1, *b*, in only four did it conform to this plan, and of these only one egg showed in the third division the regularity of pattern illustrated in Fig. 1, *c*.

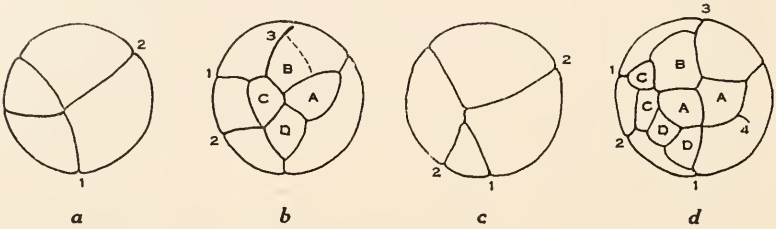

FIG. 2. Egg showing cleavage pattern resulting from inequality in the first division. Viewed from animal pole except in *c*; furrows numbered and origin of cells in *d* shown by lettering. *a*, 1 : 15 P.M., 5–12–23; *b* and *c*, 3 : 25 P.M.; *d*, 5 P.M.

Irregularity or asymmetry of cleavage in two of the ten eggs began with the first division. In one case it consisted merely in an inequality in the division of the egg (see Fig. 2, *a*); in the second division the larger of the two cells was divided more slowly than the smaller, the cleavage furrow in it reaching only thirty degrees below the egg equator when division in the smaller cell was completed. The third cleavage furrows were horizontal in three cells; in the fourth the furrow took a meridional direction, as shown in Fig. 2, *b*, in which it reaches almost to the egg equator. The vegetal hemisphere of the egg now appeared as in Fig. 2, *c*. A cleavage furrow along the dotted line of Fig. 2, *b*, subsequently cut off a micromere from cell B; this, with the division of micromeres *A*, *C*, and *D*, formed a group of seven cells of very unequal sizes arranged as in Fig. 2, *d*. The descendents of micromeres *C* and *D* formed a group of cells still distinguishable by their smaller size several hours later, in the fine-celled blastula stage; they then lay just above the egg equator. This egg subsequently completed gastrulation in the normal manner.

In the second egg to show irregularity beginning with the first division, the first furrow to appear had the position illustrated in Fig. 3, *a*. Within two hours other furrows of irregular direction had developed, giving the animal hemisphere the appearance of Fig. 3, *b*. At this time no furrows extended below the equator. Later several furrows extended into the vegetal hemisphere (Fig. 3, *c*). Fig. 3, *d* illustrates the appearance of the animal hemisphere in this egg about eight hours after the beginning of cleavage. Though continuing development for 24 hours longer and reaching a fine-celled blastula stage this egg finally died without beginning gastrulation. Its markedly atypical cleavage, therefore, may well have been the result of injury from drying, handling, or other causes.

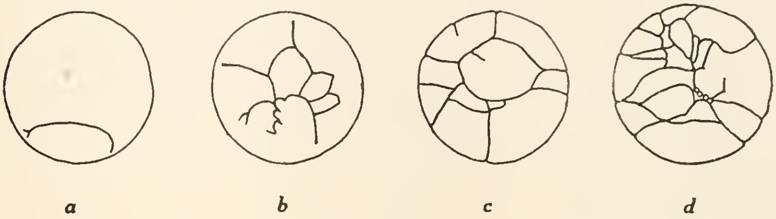

Fig. 3. Egg showing extremely irregular cleavage pattern beginning with the first division. Viewed from animal pole except in *c*. *a*, 2:45 P.M., 5-12-23; *b*, 4:45 P.M.; *c*, 8 P.M.; *d*, 10:25 P.M.

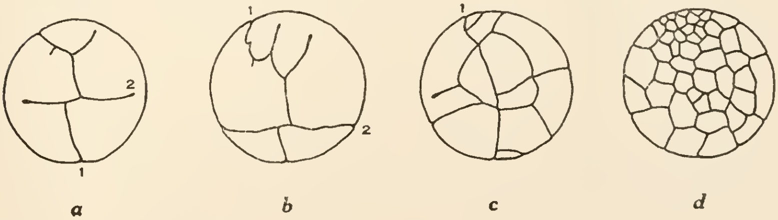

Fig. 4. Egg showing irregular cleavage pattern. Viewed from animal pole; egg in *b* is tilted to show full extent of atypical furrows. *a*, 10:25 A.M., 5-12-23; *b*, 12 M.; *c*, 1:45 P.M.; *d*, 10:45 P.M.

Another egg with almost as pronounced irregularity of cleavage as that illustrated in Fig. 3 nevertheless gave rise to a normal embryo. In this egg the first cleavage furrow developed normally, and had completely encircled the egg within three hours.

Before the usual second furrows made their appearance, a furrow was noted leading from the first a short distance above the egg equator. About a half-hour later the appearance was as in Fig. 4, *a*, with the second furrows now present. Later the egg showed other furrows in the region in which the first atypical one had made its appearance (Fig. 4, *b*) and about five hours after the onset of cleavage the animal hemisphere displayed the atypical pattern of Fig. 4, *c*. In the somewhat later blastula stage illustrated in Fig. 4, *d* this egg showed a group of distinctly smaller micromeres at and below the equator in the region of the first atypical furrow. Though this peculiarity persisted until late in cleavage this egg completed gastrulation in the normal fashion.

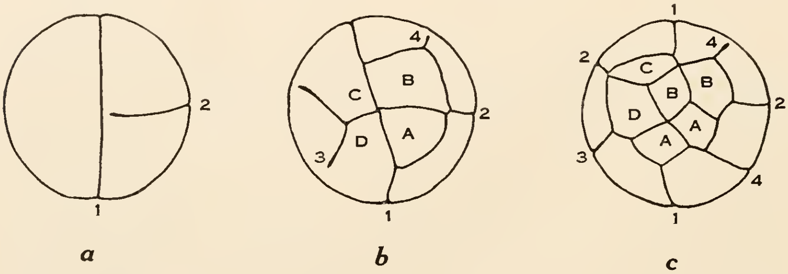

FIG. 5. Egg showing irregular cleavage pattern starting with retardation of one of second furrows. Viewed from animal pole except in *a*; furrows as numbered. *a* and *b*, 10:30 A.M., 5–12–23; *c*, 12 M. Origin of cells in *c* indicated by lettering.

Probably the most common type of irregularity initiated during the second cleavage division is that resulting from retarded or atypical formation of one of the two furrows of the set, so that one reaches the vegetal pole long in advance of the other, as illustrated in Fig. 5, *a*. The animal hemisphere of this egg pictured in Fig. 5, *b* shows that the third cleavage furrows were likewise retarded in the same half of the egg, they being completed in the half on the right while only one had appeared in the opposite hemisphere, assuming that the furrow marked 3 represents a furrow of the third set. In a stage corresponding to that of Fig. 1, *d* the polar cap of micromeres consists of but six cells (Fig. 5, *c*) instead of the eight found in the regular type of cleavage. This egg subsequently developed into a typical fine-celled blastula in which stage it was fixed for sectioning.

In many eggs the cleavage pattern becomes irregular in the course of the third division, after the first two divisions have proceeded in the regular or symmetrical fashion illustrated in Fig. 1. This irregularity may originate through retardation of division in one or more of the blastomeres, as illustrated in Fig. 6, *a*, in which only two micromeres are shown completely separated from the corresponding macromeres. Often, however, the irregularity results from the atypical direction of one or more of the cleavage furrows of the third set. In the egg shown in Fig. 6, *b*, for example, the line marked 3 represents a furrow of the third set which takes a direction parallel with the second furrow, and hence does not cut off a polar micromere in the usual fashion; the third furrow around micromere *A* is also atypical in direction, since it curves back almost to the animal pole instead of joining the first cleavage furrow nearer the equator. In the fourth cleavage

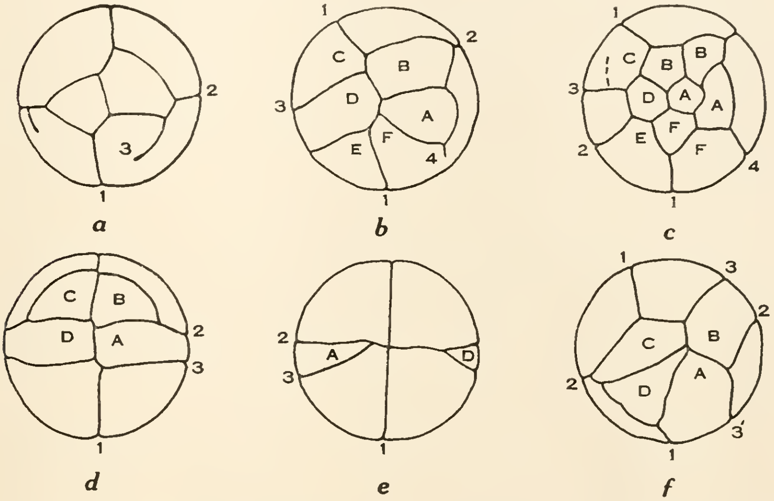

FIG. 6. Eggs showing irregularity of cleavage pattern beginning with third division, after first two have proceeded as in Fig. 1, *b*. Viewed from animal pole except in *e*; cleavage furrows as numbered. *a*, cell with all furrows of the third set horizontal, but furrows delayed in two of the blastomeres. *b* and *c*, egg with two furrows of third set atypical in direction; *b* at 11:45 A.M., *c* at 12:50 P.M. *d* and *e*, egg with two furrows of third set horizontal and the other two parallel to the second furrows in the animal hemisphere (*d*) but meeting the second furrows below the equator (*e*). *f*, egg with furrows marked 3 and 3′ atypical in direction.

division of this egg micromeres *A* and *B* were divided transversely (see Fig. 6, *c*) and micromeres were cut off from cells *D* and *F*, giving a polar cap of six micromeres, to which a seventh was added later by division of blastomere *C*. The plane of this division is indicated by a dotted line in Fig. 6, *c*. A somewhat more symmetrical pattern was exhibited by the egg shown in Fig. 6, *d;* in this egg two furrows of the third set take the usual position parallel to the equator, while the other two run parallel to the second furrows in the animal hemisphere of the egg but join these furrows in the vegetal hemisphere (Fig. 6, *e*) ; cells *A* and *D* are thus not typical polar micromeres but extend well toward the vegetal pole of the egg. Still another egg with atypical cleavage furrows of the third set is shown in Fig. 6, *f*. In this egg two furrows of somewhat typical direction had cut off the micromeres *C* and *D*, while the furrow marked 3, meeting the second furrow below the equator, had cut off a much larger cell (*B*). The furrow 3′ ultimately joined the first cleavage furrow some distance below the equator, to cut off another large cell (*A*). This egg thus resembles that of Fig. 6, *d* in possessing only two micromeres of the usual size in the eight-cell stage.

Although only a limited number of eggs were followed through early cleavage, it would seem that irregularity or asymmetry of cleavage pattern in *Hemidactylium* probably begins with the third division in the majority of cases, but with even the first or second in some cases.

Though cleavage in *Hemidactylium* follows the general plan described by Goodale ('11) for *Spelerpes* (*Eurycea*) the vegetal hemisphere of the egg is earlier divided into small cells than in the latter species. According to Goodale the cleavage furrows of the fourth set in *Spelerpes* "do not even reach the lower pole, but usually join the earlier furrows less than 45 degrees below the equator." As a result of this mode of division, the egg of *Spelerpes* in a later stage may show 130 cells visible from above and only 7 from beneath, or 400 above and 14 beneath. In *Hemidactylium* the egg is less heavily yolked than in *Spelerpes* and less markedly telolecithal. The cleavage furrows of the fourth set, though not necessarily reaching the vegetal pole, nevertheless end farther below the equator (see Fig. 1, *e*), while eggs showing 7 or 8 cells when viewed from the vegetal pole show only 25 to 30 when viewed from above. These facts indicate that the egg of *Hemidactylium* is intermediate between that of *Amblystoma* and *Spelerpes,* and far less markedly unequal in its cleavage than the relatively large eggs of *Desmognathus* (Hilton, '09) or *Cryptobranchus* (Smith, '12). In the latter forms the tendency is for the third cleavage planes to be radial or meridional in direction, and the relatively small micromeres of the animal pole arise by later divisions; in *Hemidactylium,* on the other hand, the third cleavage furrows are more frequently equatorial (latitudinal) in direction, and cut off micromeres of relatively larger size, the egg in this respect resembling that of Amblystoma.

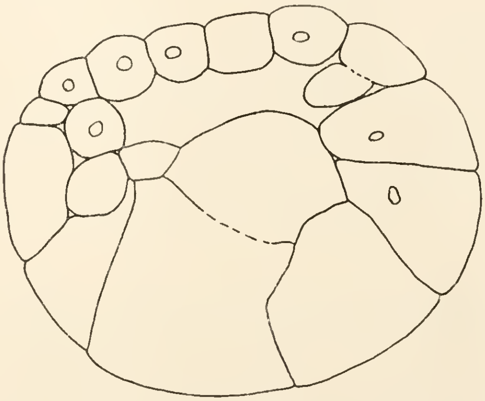

FIG. 7. Early blastula of *Hemidactylium* in vertical section. Outlined with camera lucida. × 31.

So few eggs were available that it was impossible to make a careful study of the internal changes of cleavage. Blastulæ of but two stages were sectioned. The earlier of these, represented in Fig. 7, shows a distinct segmentation cavity roofed by a single layer of cells. It is probable that the blastocoele makes its appearance early, as in the eggs of *Cryptobranchus* (Smith, '26) or *Desmognathus* (Hilton, '09), and as a distinct cavity rather than a collection of intercellular spaces such as Goodale states is frequently the condition in *Spelerpes.* Older blastulæ show a rather characteristic loose arrangement of the internal blastomeres, but a distinct blastocoele is nevertheless present.

Gastrulation and Closure of the Blastopore.

The earliest indication of gastrulation appeared about 90 hours after ovulation in the form of a crescentic depression well down in the vegetal hemisphere (Fig. 8, *a*). This crescent blastopore is bordered by a dorsal lip frequently showing a greater pigmentation than the neighboring surface. The crescent blastopore is gradually extended laterally through further invagination of cells at its lateral margins (Fig. 8, *b*) until it assumes the form of a half circumference; sometimes it may become more extensive, as in Fig. 8, *c*, but in no case was the line of invagination or overgrowth observed to become a complete circle surrounding a yolk plug. Following its period of greatest ventral extent the blastopore shortens and assumes the form of a crescent with laterally directed margins (Fig. 8, *d*); by further narrowing and elevation of its lateral portions it assumes the form shown in Fig. 8, *e*, and finally the form of an inverted crescent (Fig. 8, *f*). As the neural folds close the blastopore gradually shortens (Fig. 8, *g*)

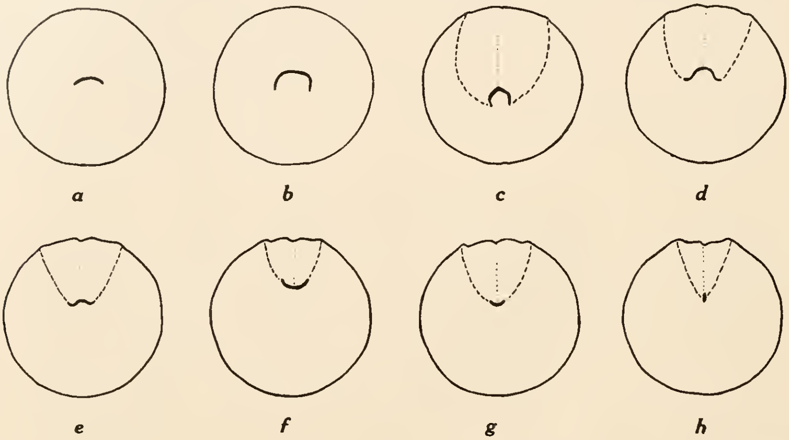

Fig. 8. Diagrams illustrating the formation and "closure" of the blastopore in *Hemidactylium*. No attempt is made to indicate exact relation of blastopore to egg equator. Margin of neural plate indicated by broken lines. *a*, early crescent blastopore; *b*, later crescent blastopore (from another egg); *c*, blastopore of *b* about 24 hours later; *d*, blastopore about 11 hours later than in *c*; *e*, later blastopore from another egg; *f*, blastopore of *e* about 9 hours later; *g*, smaller blastopore from another egg; *h*, blastopore of *g* 10 hours later, reduced to short vertical slit which persists as anus.

and finally assumes the form of a slit elongated in the direction of the body axis (Fig. 8, *h*). It does not appear that the blastoporic opening is lost but rather that it persists as the anal opening.

From the above brief account of the changes in form of the blastopore it may be seen that gastrulation in *Hemidactylium* closely resembles that in *Spelerpes* (Goodale, '11) so far as external features are concerned. Presumably the egg would show the same movements of surface material that Goodale was able to demonstrate in *Spelerpes* by the use of Nile Blue Sulphate; the egg is so lightly pigmented that it, like the egg of *Spelerpes,* might readily be used for studies of this sort. The conclusions of Goodale as to the formation of the germ layers in *Spelerpes* are probably likewise applicable to *Hemidactylium.*

Hemidactylium and *Spelerpes* are possibly unique among American Urodeles in lacking the "yolk-plug stage" characteristic of the development of Amblystoma and the *Anura.* In the larger, yolk-rich eggs of *Necturus* and *Cryptobranchus* the blastopore assumes the form of a complete circle (Smith, '12). Although Hilton claims this to be true also for *Desmognathus,* his Fig. 30 shows a late blastopore of the inverted crescentic form characteristic of the same stage in *Spelerpes* (Goodale, Pl. 1) or *Hemidactylium* (Fig. 8, *f* and *g*). Unfortunately observations on this stage of development of the eggs of *Gyrinophilus* and the Plethodons are not available; possibly in these species gastrulation would follow the pattern found in *Hemidactylium* and *Spelerpes.*

In his account of the development of *Hemidactylium,* Bishop ('18) estimates that his youngest specimen, an embryo showing well-elevated neural folds, is not more than 72 hours old. This estimate would appear to be much too low, assuming that the eggs timed by the writer had not been retarded by the conditions to which they were subjected. In these eggs the early crescent blastopore was first observed about 90 hours after ovulation, and the neural folds did not close until from 60 to 80 hours following this stage, or at from 150 to 175 hours after ovulation. Since temperature determines to a great extent the rate of the developmental processes, and since the laboratory temperature averaged higher than that of the normal environment, it is probable that the eggs observed developed at least as rapidly as would eggs in the field.

For stages of development later than those of Fig. 8 the reader is referred to the excellent sketches published by Bishop ('18). Transections of older embryos of this species are figured in a previous report dealing with the primordial germ cells (Humphrey, '25).

SUMMARY AND CONCLUSIONS.

1. The egg complement of the *Hemidactylium* female probably requires a period of several hours for its deposition, since the expulsion of a single egg occupies several minutes.

2. The observed position of the body of the female during ovulation (ventral surface upmost) may be of value in preventing scattering of the eggs, since they are thus supported upon the body of the female as well as by adherence to neighboring sphagnum or other eggs.

3. Under laboratory conditions cleavage begins in from ten to fifteen hours after ovulation; it is of the unequal holoblastic type. The first and second cleavage furrows tend to be meridional in direction. The furrows of the third set are frequently horizontal (latitudinal) and lie well above the egg equator, but in many cases they take a direction parallel with the first or second furrows in the animal hemisphere and meet these furrows at some point below the equator. A well developed blastocoele is very early present.

4. Irregularity in cleavage is to be expected in all eggs after the third division. Only occasionally do the eggs show symmerical eight-cell stages, due to the fact that irregularity or asymmetry in cleavage may begin with any of the first three divisions.

5. The vegetal hemisphere of the egg undergoes more division than in the eggs of *Spelerpes, Desmognathus,* or *Cryptobranchus.* In this respect it resembles the egg of *Amblystoma.*

6. Gastrulation begins about 90 hours after ovulation with the formation of the usual crescent blastopore. No ventral lip develops and no yolk plug is formed. The crescent blastopore shortens and becomes inverted, the horns of the crescent pointing upward, and finally closes to a vertical slit which becomes the anus.

7. Closure of the neural folds occurs in from 150 to 175 hours after ovulation. The further course of development has been described by Bishop ('18).

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FERTILE TERMITE SOLDIERS.

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In two earlier studies (Heath, '03, '27) attention was called to the fact that in the termite genus *Termopsis,* represented in California by two species (*T. nevadensis* and *T. angusticollis*), occasional individuals occur which externally resemble a soldier and yet are sexually mature. In the typical soldier the head is distinctly longer than broad, whereas in the fertile type the two diameters are the same or nearly so. Furthermore the jaws of both project far beyond the labrum, and are built upon essentially the same plan. Hence, unless it is decided that they belong to a special caste, these unusual insects must be looked upon merely as soldiers with fully developed sex organs.

Up to the time when the manuscript of the later paper went to press only four of this type of termite had been discovered. Since then fourteen additional individuals have been collected, and it now appears that they may prove to be of rather frequent occurrence. All of these later acquisitions were living in essentially the same conditions. A limb of a Monterey pine (*Pinus radiata*), ten or fifteen feet in length, and evidently occupied by a colony of *T. nevadensis* throughout its entire extent, had broken off near its base, and in falling had broken into several fragments. When the weathering of these, and the depth they had sunk into the leaf mold indicated a sojourn of several months each fragment was carefully opened. A large number of such colonies were examined, and while in most instances complemental royal forms were present, those with soldier-like appearance existed in approximately twelve per cent. of the communities.

In addition to their relatively small heads the behavior of these fertile soldiers is unmistakable. Unlike the typical soldier, which moves about from place to place in the observation nest, the fertile type remains in close association with the normal complemental insects. Also the females are readily distinguished by their distended abdomens. Conjugation was witnessed on six occasions where the soldier paired with an individual of the usual neoteinic type. No especial attempt was made to observe the actual egg-laying process, since this had been observed on two former occasions. Dissections, however, of six specimens showed the sex organs to be well developed. In the text figure, for example, a specimen of female complemental soldier is compared with a primary queen from a colony of *T. nevadensis* two and one half years old. In the first named individual (*A*) there were seven fully-formed ova, including one in the oviduct (not represented), while in the other

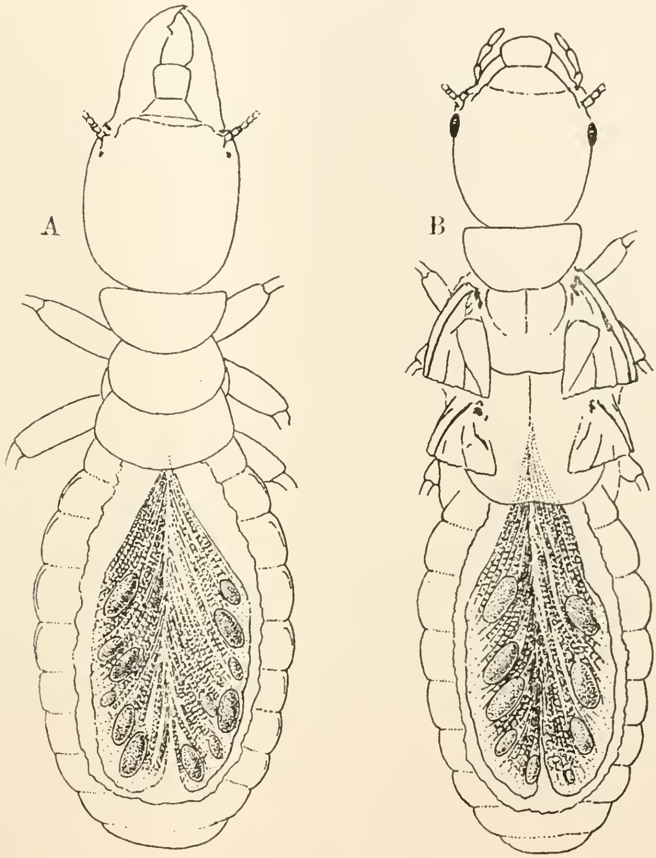

Fig. 1.

specimen the ovary contained eight fully-developed eggs. In addition there were approximately the same number of developing ova in both insects.

In a former account (Heath, '27) some observations were recorded which indicate that the complemental royal forms, at least in the genus *Termopsis*, are fertile soldier nymphs. The fertile soldier also points to the same conclusion. Under normal circumstances it appears that in an orphaned colony insects of this type appear as a rule to be in the penultimate instar, and yet there are occasional specimens which give evidence of belonging to the antepenultimate stage. Also it appears to be true that in some instances the sex gland stimulus may be applied to individuals which have progressed so far along the path to complete soldier development that with the functional activity of the gonad there is a slight suppression only of those characters which normally belong to the fully-developed soldier. This seems to be a reasonable explanation of the origin of these individuals, which are thus seen to combine the activities of the usual complemental royal insect together with many of the structural features of the typical soldier.

Up to the present time no essential differences have been discovered between the offspring of the primary royal pair and those of the complemental royal forms including the fertile soldier. The data relating to this last named category, however, are based upon the study of two colonies only. A report upon a more extensive series of experiments, now being conducted, will be made at a later date.

REACTIONS OF THE CYPRID LARVÆ OF BARNACLES AT THE TIME OF ATTACHMENT.[1]

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Knowledge of the actual process of attachment and of the reaction of the organism immediately preceding this act is very meager for many of the sessile marine organisms. As a large number of these forms cause fouling on ship's bottoms, a careful study of some has been made by the author as part of a study to determine the nature and extent of fouling on ship's bottoms.

The activities of the tadpole larvæ of several species of tunicates have been carefully studied by Caswell Grave (1921–1923). He found that after a free-swimming period which varied from a few minutes to several hours, the larvæ invariably became negative to light and attached by means of adhesive papillæ. The factors which determined when the organisms attached appeared to be related to the physiological state of the organism, while the place of attachment is probably a matter of chance, although the reactions to shadows may determine this in some degree.

Nelson (1924) found "that in the oyster the larvæ 'test out,' by means of their feet, an appreciable area of solid surface before actually attaching, which is preceded by circling movements." It had long been supposed that these larvæ had simply settled and the lucky ones survived.

The only record of attachment of larval barnacles of which the author is aware is found in Darwin (1851). He states that "the larva fixes itself with its sternal surface parallel and close to the surface of attachment, and the antennæ become cemented to it; if the cirripede, after its metamorphosis had remained in this position, the cirri could not have been exserted, or only against the surface of attachment; but there is a special provision that the young cirripede shall immediately assume its proper position at right angles to the position which it held whilst within the larva, namely with its posterior end upwards. This is effected in a singular manner by the exuviation of the great compound eyes, which we have seen are fastened to the outer arms of the double UU-like, sternal apodemes; these together with the eyes stretch transversely across, and internally far up into the body of the larva; and, as the whole has to be rejected or moulted, the membrane of the peduncle of the young cirripede has necessarily to be formed with a wide and deep inward fold, extending transversely across it; this when stretched open, after the exuviation of the larval carapace and apodemes, necessarily cause the sternal side of the peduncle to be longer than the dorsal, and, as a consequence, gives to the young cirripede its normal position, at right angles to that of the larva when first attached."

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[1] Published by permission of the U. S. Commissioner of Fisheries. Experiments were performed at the U. S. Biological Station at Beaufort, N. C.

Barnacles attach to a great many materials and under very diverse environmental conditions. Darwin (1853) stated that " sessile cirripedes adhere to all sorts of objects, floating and fixed, animal and vegetable, living and dead, organic and inorganic," and that " living mollusca are the most frequent objects of attachment." The following list of materials will serve to give some idea of the varied array of materials on which the writer has seen barnacles attached: wood, glass, rubber, beeswax, shells of more than thirty varieties, rocks of many sorts, rope, cloth, leather, metals of many kinds including iron, copper, zinc, lead, tin, aluminum, and to many alloys as brass and bronze, turtles, crabs, lobsters, fucus fronds, bamboo, zostera, sponges, corals, echinoderms, bryozoa, tunicates, and several types of cetacea. It is thus apparent that barnacles attach to almost every type of material submerged in sea-water, which is sufficiently large and permanent to afford a place of growth.

But although barnacles attach to all these materials, there is nevertheless a high degree of specialization among them. Thus *Balanus galeatus* is found only on certain gorgonian corals; *Coronula diadema* only on the humpback whales (*Megaptera*); *Cryptolepas rachianecti* only on the Californian grey whale (*Rhachianectis glaucus*). *Octolasmis mulleri* is found in the gill chambers of several crabs, while *Octolasmis geryonphila* is found only in the gill chamber of a single type of crab (*Geryon quin-*

quedens). *Chelonibia patula* grows only on the carapace of certain crabs (and on *Limulus*) while *Chelonibia testubinaria* grows only on the carapaces of turtles.

Although many thus show a high degree of specialization as regards their habitat, yet within a single genus one often finds wide extremes. *Balanus amphitrite* is perhaps the most widely distributed barnacle existing today, with a world wide distribution, being found in all tropical and warm temperate waters. It attaches to wood, to rocks, to fucus fronds, to rubber, to metals of many sorts and to shells as well as living molluscs of many kinds. In contrast to this, *Balanus galeatus* attaches only to a limited number of gorgonian corals and is typically a West Indian species, although a few specimens have recently been found from Southern California.

The larvæ of all barnacles are hatched as nauplei, the eggs being carried within the mantle cavity of the adult until hatched. These nauplei moult from one to three times during the course of the first week or ten days, changing slightly with each moult into forms known as metanauplei and then metamorphose into an entirely different type of organism called the cypris larvæ or cyprid. The cyprids of all barnacles are bilaterally symmetrical organisms about four times as long as high and often ten times as long as broad. These proportions vary depending upon the genus of the barnacles but the species of any given genus seem to vary but little in this regard.

This free-swimming period as cyprids appears to vary greatly with different forms of barnacles. *Balanus amphitrite* has been observed to attach within seven days after hatching, while *Chthamalus fragilis* probably attaches within four days. *Balanus balanoides,* on the other hand, has a much more extended larval life and apparently does not attach for from 10 to 12 weeks after liberation.

These cyprid larvæ propel themselves through water by means of sudden backward movements of their appendages. These movements occur with great rapidity so that it is quite impossible to see this reaction unless the organisms are swimming very slowly.

They are aided during their pelagic life by the fact that they possess great globules of a fatty substance in the anterior portion of their bodies. That this is clearly a lipoid in its character has been demonstrated by its reaction with Congo red and with osmic acid. During much of this period this mass of liquid substance seems to act as a buoy holding the larvæ near the surface film. But as the free swimming period draws to a close this substance disappears. It seems probable accordingly that the length of the free-swimming period is dependent upon the amount of this substance originally stored and its rate of disappearance.

Throughout this period of pelagic life the cyprids often swim on their sides, that is, with their oral surfaces on a horizontal plane with their dorsal surfaces. This holds good especially if the organisms are in the surface film at the surface of the water or if they rest on some object at the bottom. They will frequently propel themselves for some time through a considerable distance while in this position. It has also been observed that they usually are found when in this position to have their morphologically right side turned downward while their left side is uppermost.

The actual process of attachment occurs, as suggested above, after a free-swimming period of from three days to two weeks (longer for some species). The cyprids of *Balanus improvisus* and *Balanus amphitrite* have been kept under observation for ten or eleven days at the end of which time some were found to have attached but many were still active.

When the internal physiological conditions necessary for attachment are present, conditions apparently correlated with the "lipoid" content of the organisms, the larvæ have been observed on many occasions to "walk" on the substratum apparently "hunting" a place for attachment. This remarkable performance is accomplished by alternate attachment and release of the adhesive tips of the antennæ combined with a "push" from the set of appendages.

By reference to Fig. 1, it will be seen that when one of the antennæ (*a*–2) is released the appendages (*ap*) straighten out, throwing the larva forward. The released antenna (*a*–2) then attaches, while the appendages are withdrawn, and with the next move the antenna (*a*–1) is released.

In this manner these organisms have been observed to traverse a distance of more than twelve mm. (about ½ inch) and have been

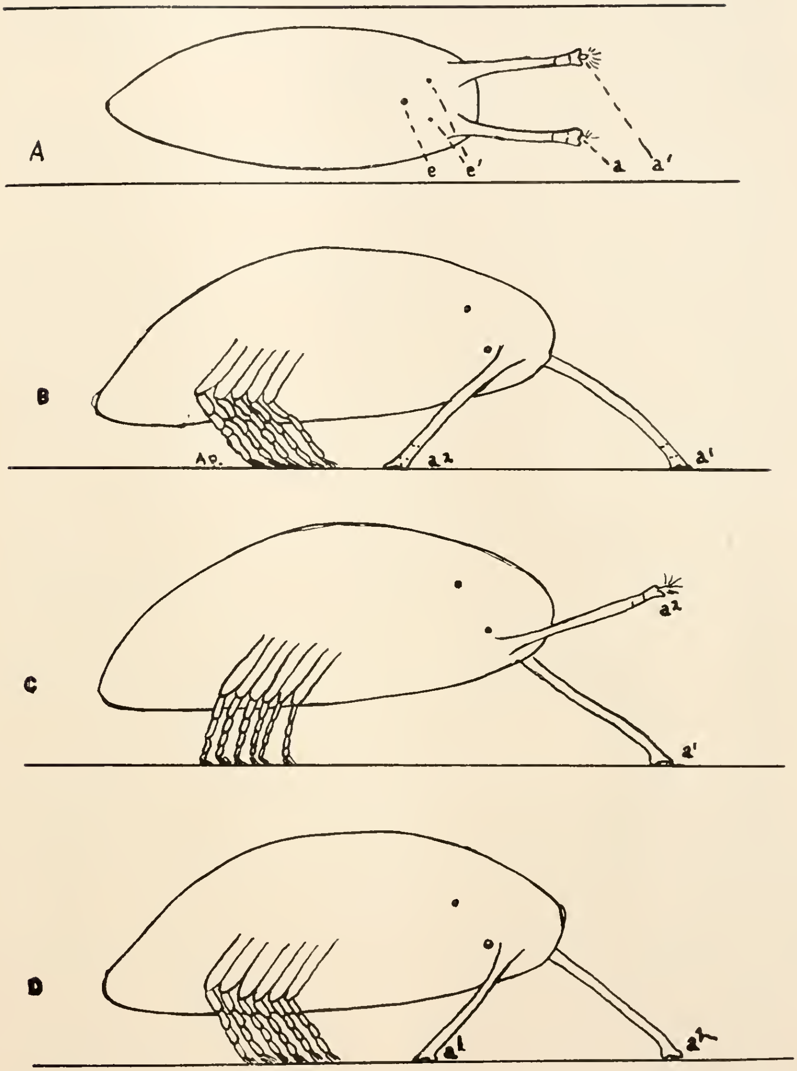

FIG. 1. To illustrate the movements of barnacle larvæ at the time of attaching. *A:* ventral view to show antennæ, a' and a^1; median eye, *e*, and the paired lateral eyes, c'. *B–D,* lateral views of cyprid larva, showing method of locomotion at time of "selecting" a place for attachment.

seen to "test" different areas for a period of more than one hour before finally attaching. Frequently it has been observed that the antennæ appear to adhere so firmly that the larva "kicks" for eight or ten times before releasing its hold.

On several occasions the writer has been fortunate in seeing the actual process of metamorphosis while observing through a microscope giving a magnification of about one hundred and fifty diameters. It was then seen that after attachment by means of the antennæ, the organisms "kick vigorously" for some time, but without effecting release. The animal then appears to settle down as soon as attachment was effected, in the region of the appendages. Metamorphosis now occurred. The two-valved shell of the cyprid stage was thrown off, including the exoskeleton of the appendages (often also the paired eyes). From the resulting almost amorphous mass the young barnacle is soon made out. A secretion is continually laid down on the formerly ventral surface, and the rudiments of a coating (the future shell) appear around the sides of the mass. After one hour the "head" and appendages of the animal can be seen in an inverted condition. Whereas when attached the appendages extend downward, they now extend upward and the mouth parts too have changed their position; this reversal in position occurring as described by Darwin as quoted above.

No opercular valves appear until after six hours, while the separate plates of the shell do not appear until more than twelve hours after metamorphosis, in any of the observed cases of *Bal. amphitrite, Bal. improvisus,* or *Chthamalus fragilis.*

In these types the plates appear as four units taking the place of the original apparently chitinous material which protected the organism during the early stages of metamorphosis.

The cyprid larvæ of barnacles are also sensitive to light and it has been shown (Visscher and Luce, 1928) that they are stimulated to a much greater degree by a given amount of energy in the field of green than by like amounts of energy in other fields of the spectrum. These larvæ are usually positive in their reactions but in the later stages they are very erratic and may not show either positive or negative reactions for a considerable period of time. However, at the time of attachment, although still sensitive to light and especially to wave-lengths of 470–500 $\mu\mu$ they become decidedly negative and move away from the source of stimulation.

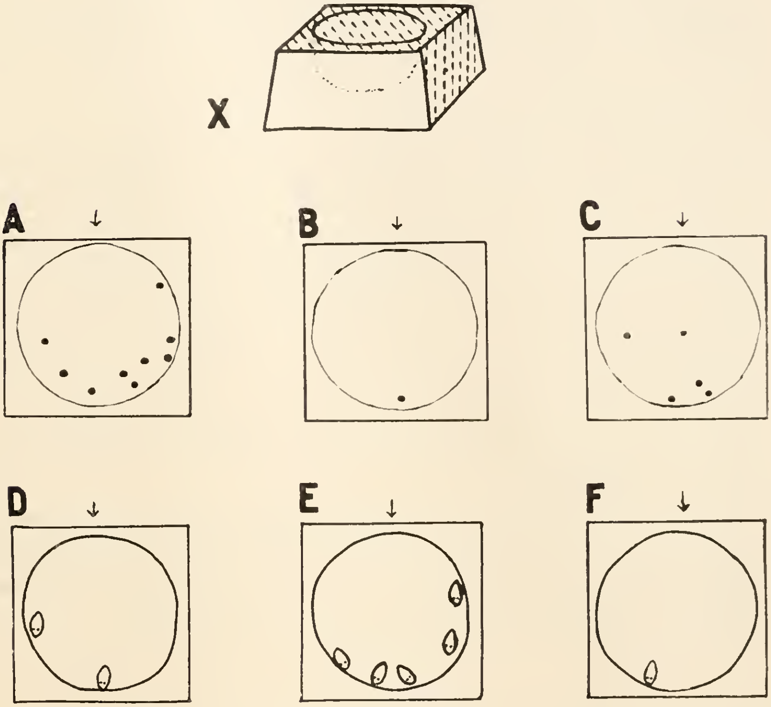

Fig. 2. To illustrate effect of light at time of attachment of larval barnacles. *X*, glass dish, 4 cm. square, by 1.5 cm. in height, with concave depression 2½ cm. in diameter and 1 cm. in depth, covered with heavy black paper on five of its six sides as indicated. *A–C*, and *D–F*, two series of tests, contained barnacle larvæ, in which each container was covered as in *X*, and with the exposed side toward the window as indicated by the arrow. Only those larvæ which had attached and metamorphosed are indicated.

It has been impossible to select cyprid larvæ of known age, but the following experiment will demonstrate this reaction. A number of cyprid larvæ were isolated from a collection of tow made in the tidal channel in front of the laboratory island. From the original lot those with the least amount of "oil droplets" were selected and placed in small glass dishes resembling salt cellars which were surrounded on five of the six sides with a dull black paper (Fig. 2). These were then set on a table in front of a large north window with the uncovered side of the dish toward the window.

In the first series of three experiments twelve cyprid larvæ of *Bal. improvisus* were placed in *A,* three cyprids of *Chthamalus fragilis* were placed in *B,* and between twenty and thirty mixed forms were placed in *C.*

By referring to Fig. 2, it will be seen that attachment occurred almost without exception on the side of the dish away from the source of light. It was observed at this time that many of these young barnacles were definitely oriented with their anterior ends (containing their eyes) decidedly away from the light side of the dish. In a second series of experiments *D, E,* and *F* (Fig. 2) this feature is even more clearly seen. Although these experiments were repeated some two weeks later with large numbers of individuals only a very few were observed to attach, and the results were no more conclusive than those shown in the first two series of experiments.

It can be clearly seen from these experiments that for the two types of barnacles which were tested, light is an important factor in determining the point of attachment, and that they orient with the anterior end directed away from the source of light.

CONCLUSIONS.

Barnacle larvæ are sensitive to light at the time of attachment and for the three species tested, are negative to light at this time. It has also been demonstrated that barnacle larvæ do not attach at random, *i.e.,* merely by chance, but that they apparently " test out " the surface before attaching, in which process they have been observed to spend a very considerable period. The antennæ are evidently the " feelers " as well as the final " hold-fasts " and once definitely attached a secretion is deposited which protects the young barnacle from the beginning of its life as a sessile organism.

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REACTIONS OF THE CYPRID LARVÆ OF BARNACLES TO LIGHT WITH SPECIAL REFERENCE TO SPECTRAL COLORS.[1]

J. PAUL VISSCHER AND ROBERT H. LUCE.

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INTRODUCTION.

It is well known that the larvæ of barnacles as well as the larvæ of almost all sessile marine organisms are, at times, sensitive to light and are either photopositive or photonegative. This fact has led to their use for experimental purposes, but the use of the larval forms of barnacles in such experiments has been confined to the earliest free swimming stage call the *Nauplius*. No records have been found of the use of the later larval stage, the cyprid larva in experiments with light.

Since barnacles are the most important of the various types of organisms which cause fouling (cf. Visscher, 1928) and since barnacles attach while in the cyprid stage and subsequently metamorphose into the adult form and since it had been observed on ship's bottoms that barnacles were frequently most numerous on the shaded area of the hull, it seemed important to ascertain the stimulating efficiency of light of different wave-lengths in regard to the cyprid larvæ of barnacles.

In previous work on the reactions of various organisms to color, much confusion has arisen from the fact that the apparatus used was neither standardized nor calibrated. Where spectral transmissions were known, there was frequently no attempt to balance the radiant energies. The only occurrences of the use of different spectral colors with balanced radiant energies are those of Pergens (1899), Blauuw (1908), Laurens (1911), Day (1911), and Gross (1913).

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[1] Published by permission of the U. S. Commissioner of Fisheries. Experiments were performed at the U. S. Biological Station at Beaufort, N. C.

The most common error in the earlier work was that of ascribing the effect of colored light to its wave-length, and ignoring the effect of intensity. Pergens was the first to recognize the difference between wave-length and intensity. Working with a spectrometer he found that the energy of the blue end of the spectrum was too small to be measured. He then used light filters of colored glass, and measured the light intensity with a Ritchie photometer. Pergens failed, however, to eliminate the effect of the infra red transmitted by the filters.

Laurens, Day and Gross used apparatus which was essentially alike. The light was diffracted and balanced and the band of transmission was either narrowed or widened until the deflection of the galvanometer was the same for all the colors. While this work was done with colors of high purity and balanced radiant energies, it cannot give more than a relative idea as to the stimulating efficiency of parts of the spectrum, as only four limited regions were employed.

Mast (1917) used a Hilger spectrometer and a Lummer-Brodhun rotating sector and was able to determine with great accuracy the relative stimulating efficiency of the various parts of the spectrum. While this method allows a direct study of the effectiveness of spectral colors, it appeared to be limited in its application to organisms which, in a single beam of light travel parallel to the rays and do not deflect to either side and consequently could not be used with cyprid larvæ. The intensity of light obtainable by this method was believed to be too limited for adequate work on the cyprid larvæ of barnacles. For these reasons and others, to be discussed later, it was considered inadvisable to use this method in the following work.

Materials and Methods.

The larval life of the barnacle is divided, with a very few exceptions, into two distinct stages, the nauplius, and the cyprid. The cyprid stage is characterized by the presence of a large bivalve shell, giving it a general resemblance to certain ostracods. This resemblance, however, is superficial and does not extend to the limb structure or internal anatomy. All of the appendages of the adult are present; the antennules protrude from between the valves of the carapace; there are six pairs of thoracic limbs, but no abdominal appendages. The unpaired eye of the nauplius is usually still present, and in addition there is a pair of large compound eyes.

Cyprids of various species of barnacles differ from each other in the size and proportions of the carapace, and in the presence of certain characteristic bodies within the carapace. During the time of year in which this work was done, three main types of cyprids predominated. These types were of two distinct sizes. The smaller size was .51 mm. long, .225 mm. high, and .20 mm. wide, and was found by subsequent metamorphosis to be the cyprid of *Chthamalus fragilis*. The larger type was made up of two groups which were outwardly very similar, being .65 mm. long, .24 mm. high, and .20 mm. wide. These groups could only be distinguished by the presence of certain granules in the carapace. One of these was determined by subsequent metamorphosis to be *Balanus improvisus*, and the second was the cyprid of *B. amphitrite*.

Balanus improvisus and *B. amphitrite* are known to breed most abundantly at Beaufort during June and July, and large numbers of their nauplii and cyprids are to be found at that time. *Chthamalus fragilis* has been observed to have a more extended breeding period, apparently running from May into late September. *Balanus eburneus* is abundant at Beaufort, but as far as is known, no cyprids of this species were used in the experiments.

Cyprids were obtained by towing with a number 12 mesh net. They were picked out of the tow by means of a capillary pipette, then washed in clear sea water and placed in the aquarium. During the summer of 1924 cyprids were not numerous but during the summer of 1925 cyprids were very abundant, comprising at times in late June and early July a large part of the plankton. This abundance allowed a selection of material, and in consequence most of the work was carried out with the cyprids of *B. improvisus* and *B. amphitrite*. Cyprids appear to be most abundant on an incoming tide and at flood tide. This is also true for the nauplii during June when they were most abundant. There is a marked diurnal migration, so that tows during the day must be made with the net about eight feet below the surface, this being slightly less than the minimum depth of the channel. The time of exposure to sunlight exerts a marked influence on the activity of those cyprids which remain at the surface. Cyprids taken from a surface tow during the morning are fairly active, but those taken from a surface tow during the late afternoon are inactive, and could not be used.

The apparatus used in the following experiments consisted of 100 watt, gas-filled, tungsten lamps, two copper sulphate cells, and thirteen monochromatic light filters.

In selecting the filters, an attempt was made to use only those having a narrow transmission band. This is somewhat difficult to obtain for the yellow and blue-violet. Wratten filters were available for most of the needed colors, but these filters are not wholly stable under the climatic conditions found at Beaufort. At the suggestion of Dr. K. S. Gibson of the Bureau of Standards, we were able to obtain a number of filters from glasses manufactured by the Corning Glass Works, which company very kindly supplied them for our use. These glasses were ground to thicknesses giving transmissions in accordance with the curves shown in Bureau of Standard Technologic Paper 148. The glasses are more satisfactory than Wratten filters because of their permanency under all climatic conditions. However, Corning glasses were not available for certain regions of the spectrum, so four Wratten filters were used, making a series of thirteen filters covering the whole visible spectrum. By referring to figure No. 1 a list of all "Corning" filters with their curves of transmission can be found. Table I. is a summarization of the curves for all filters used giving the limits of transmission and the dominant wave-length of each filter.

The difficulty encountered in obtaining a deep blue or ultraviolet arises from the fact that all the filters in this region transmit some of the red end of the spectrum. No filter was available to block out the red without also cutting off some of the blue or ultraviolet. Since red is relatively feeble in stimulating effect, it was judged best to use the blue and untraviolet filters as they were, and to attempt a correction for the presence of the red later. We were not able to obtain a yellow filter with a sharply limited transmission band, since ordinary yellow filters merely cut off a portion of the blue end of the spectrum. Perhaps the best yellow would be produced by a combination of Corning glasses G36 (curve 38) and 401Z (curve 59) as given in Bureau Standards Paper No. 148. Unfortunately, however, this combination was not available to us at the time. The balancing of the radiant energies for all filters was done by the Bureau of Standards, by the use of an apparatus based upon the radiomicrometer of Boys.[1]

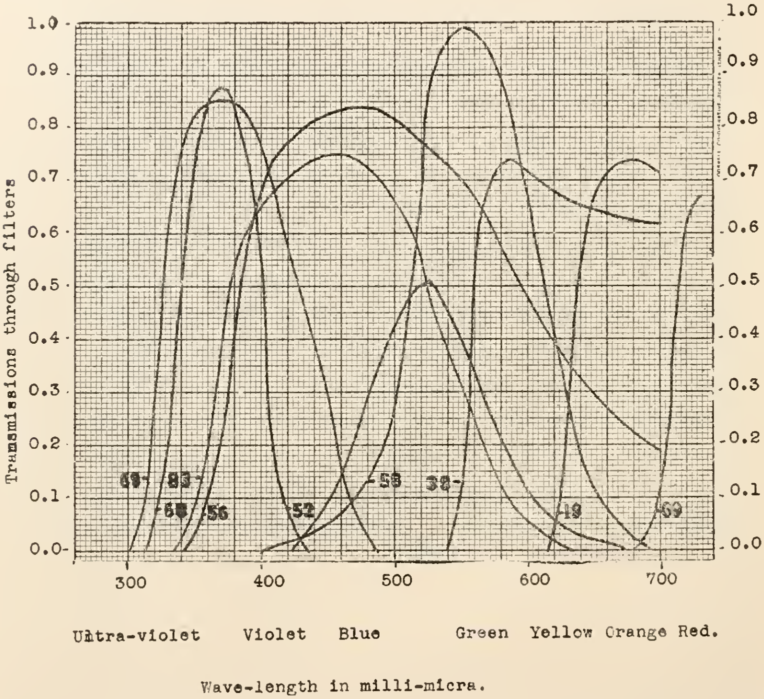

FIG. 1. Curves to illustrate the range of light energy transmitted by the "Corning" glass filters used to test the relative stimulating efficiency of spectral colors. (From the U. S. Bureau of Standards Technologic Papers No. 148.)

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[1] The authors are grateful to the Director of the Bureau of Standards and also to Dr. W. W. Coblentz for their kindness in calibrating all filters and lamps used for these experiments.

TABLE I.

LIST OF FILTERS USED IN EXPERIMENTS, SHOWING THEIR TOTAL SPECTRAL TRANSMISSION, AND THEIR DOMINANT WAVE-LENGTHS.

Filter.	Total Transmission	Dominant Wave Length.
Ultra C 83	315–428 $\mu\mu$ 609–Red End	355 $\mu\mu$
Purple C 69	310–485 $\mu\mu$ 690–Red End	370 $\mu\mu$
Purple W 35	300–475 $\mu\mu$ 650–700 $\mu\mu$	420 $\mu\mu$
Blue W 49	400–510 $\mu\mu$	440 $\mu\mu$
Blue C 60	335–640 $\mu\mu$	460 $\mu\mu$
Blue C 59	335–690 $\mu\mu$	480 $\mu\mu$
Blue Green C 56	340–700 $\mu\mu$	505 $\mu\mu$
Green C 52	425–670 $\mu\mu$	530 $\mu\mu$
Green W 58	485–635 $\mu\mu$	540 $\mu\mu$
Yellow W 15	500–700 $\mu\mu$	590 $\mu\mu$
Orange W 22	545–700 $\mu\mu$	620 $\mu\mu$
Orange C 38	540–Red End	640 $\mu\mu$
Red C 19	620–Red End	700 $\mu\mu$

The letter "C" after a filter denotes a Corning glass filter. The numbers after the Corning glasses refer to the transmission curves shown in Bureau of Standards Technologic Paper 148. The letter "W" denotes a Wratten filter, and the number refers to the transmission curves found in the booklet "Wratten Filters" published by the Eastman Kodak Company.

Each lamp to be calibrated was set up with the center of the curved filament facing a radiometer. The radiometer was placed on a scale graduated in centimeters, and could be moved toward or away from the light. Connected with the radiometer was a Coblentz modification of Thompson's galvanometer. This was hung on the wall, fifteen feet from the radiometer, and at right angles to it. The lamps used were 100 watt, 115 volt, gas-filled, Mazda lamps and during the test each lamp carried 109.3 volts and 0.87 amperes. In these experiments the infra-red was absorbed by copper sulphate cells. Each cell was two centimeters thick, and consisted of two pieces of optical glass cemented to a glass ring, five centimeters in diameter. The cells were filled with a solution consisting of 57 grams $CuSO_4$ in two liters of water. One of these cells was placed in front of each lamp tested. The radiometer was then moved back and forth until the galvanometer showed a deflection of one centimeter. This was taken as a standard deflection. A filter was then placed before the lamp and the galvanometer again brought to a deflection of one cm. Such balancing of the radiant energies, therefore, eliminates the necessity of correcting for the unequal distribution of energy in the spectrum of the lamp. The result of any experiment conducted with these balanced filters represents, therefore, the actual stimulating efficiency of the specific wave-length of the light, and is not in any way influenced by a difference in light intensities.

The apparatus was set up so that two beams of light crossed at right angles (Fig. 2). The lamps were enclosed in light-proof boxes each having an opening five centimeters square. A copper sulphate cell was placed in front of each of these openings. Each box was readily moved, and the center of the crossing of the two beams was used as the zero point from which to measure the position of the light. The aquarium in which the organisms were placed was made of the best quality slide-glass, and was 35 mm. square and 10 mm. deep. This aquarium was placed on a stand at the center of the two cross beams. Underneath this aquarium was placed a small microscope lamp which could be lighted momentarily so that the cyprids could be counted more readily. Two screens were placed just outside of the aquarium stand, one of these had an opening admitting white light, and the other had a slot in which the filters were placed. In practice, the lamp giving the white beam was placed permanently with the center of the filament at a point 119.2 cm. distant from the center of the two beams of light while the lamp used with the filters was moved to such distances as to compensate for the energy transmission value as previously determined for each filter.

Ten cyprids were placed in each aquarium in carrying out an experiment. They were allowed to become dark adapted, and were then given a one minute exposure to light. At the end of this time the cyprids in the white light, in the filter light, and in the corner between the two light beams, were counted. These numbers thus obtained were recorded in a table of four columns under the heads of White, Corner, Filter, and Non-reacting. A dark period of about twenty seconds was given between trials. Three trials were made with each filter, after which the filters

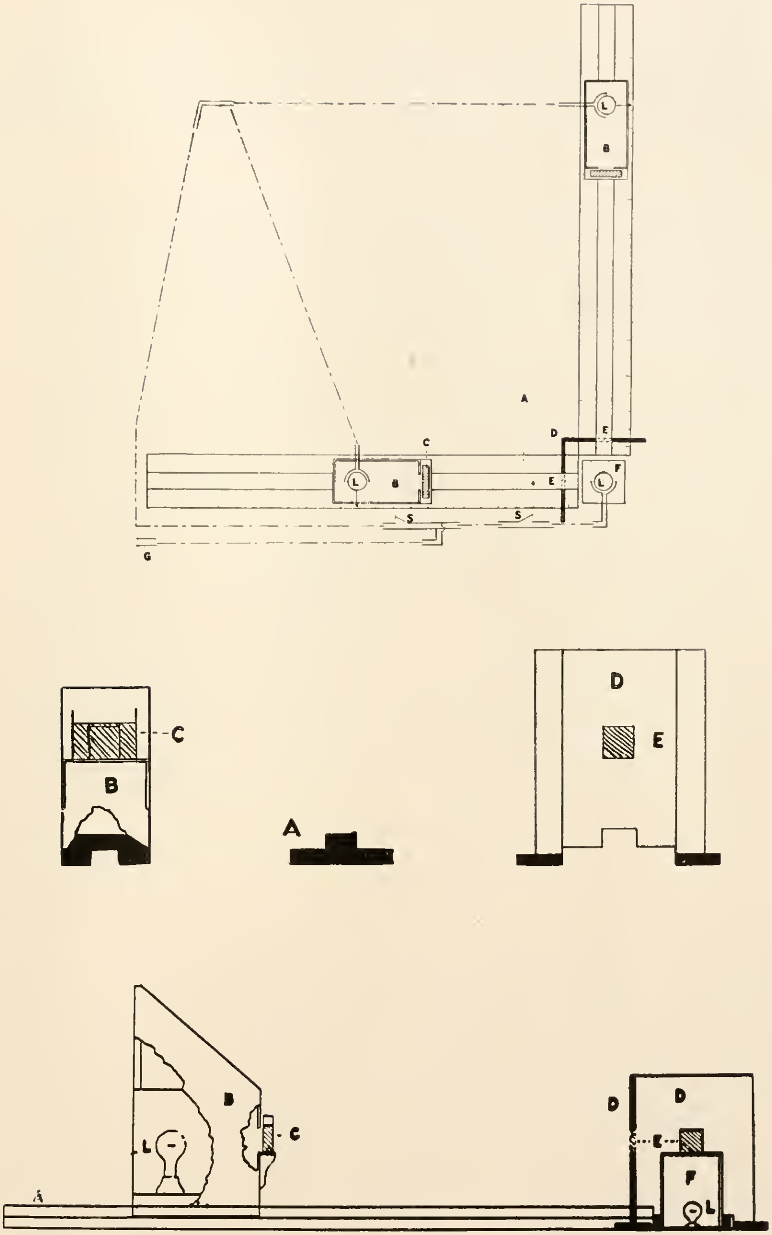

Fig. 2. To illustrate apparatus used to test the relative stimulating efficiency of equal amounts of light energy, in known and limited fields of the spectrum, upon the cyprid larvæ of barnacles. *A,* Track for boxes (*B*) containing the electric bulbs (*L*). *C,* copper sulphate filters in front of square opening in light box (*B*). *D,* screen holding filters (*E*) and surrounding aquarium stand (*F*). *S,* switches for control of lights. *G,* feed line for electric current.

were changed. The same cyprids as a rule could be used for the whole series of filters, though certain factors modified their behavior.

BEHAVIOR OF CYPRIDS.

Cyprids give definite reactions to light for two to three hours, but after that the number of non-reactants increases rapidly. They must also be dark adapted in order to secure definite movements toward the light, but apparently there are limits to this dark adaptation. It was noted during the experiments that if the cyprids were left in total darkness for periods of thirty to sixty minutes, that on resuming the experiments there were no reactants during the first exposure. In the second exposure there were a few reactants, but several exposures were required before the usual number of reactants were obtained. This, however, can be demonstrated more strikingly with nauplii, where, in several instances, ten exposures were required before all of the nauplii were reacting. The effect of a shorter dark period is also well marked. A dark period of one minute was used during the earlier experiments. By varying this rest period, however, and counting the number of reactants and non-reactants, it was found that a rest period of about twenty seconds gave the smallest number of non-reactants. Such a period varies somewhat as the experiment progresses, for a shorter period can be used in the first part of the experiment, and a slightly longer period is necessary toward the end.

Cyprids usually lie upon either side when not in motion. Movement is usually on the side, and is brought about by rapid and powerful kicks of the thoracic appendages. The movement is rapid and usually in a wide arc, but sometimes in a straight line. It is not at all unusual for them to turn about in a complete circle or series of circles before shooting off toward the light. The position of the antennæ apparently has much to do in determining the direction of movement, the movement being in a wide arc or fairly straight line when the antennæ are withdrawn into the carapace, and circular when they are extended. The cyprids may either sink down and lie quietly on the bottom after striking the side of the aquarium, or they may move back and forth along the side of the dish in a series of jerky movements. This uncertainty in their movements makes it necessary to have a definite time of exposure, and also necessitates a rapid counting of those collected in each light.

The presence of small oil droplets in the anterior end of the cyprids modifies their behavior to a considerable extent. These droplets are usually present, and are found either in the anterior end or along the dorsal side. Their size may apparently be taken as an indication of the age of the cyprid. Cyprids about to attach have very small drops, are sluggish, and move about on the bottom of the dish with a walking movement of the antennæ and a push or slow kick of the thoracic appendages. Those in which the oil droplets are large, often become caught in the surface film. When this occurs it is difficult to get them out, and while they may be very active, they cannot react definitely toward the light. Their reaction consists of spasmodic kicks and short movements toward the light, but these movements soon cease and they drop back passively toward the center of the dish. If two or more cyprids get into the surface film, they cluster together and are of no further value in the experiment. In such cases they were removed and replaced by fresh organisms. The cyprids which are the most favorable for study are those which are too old to get in the surface film, and which are not ready for attachment.

RESULTS.

In Table II. are shown the results of twenty series of experiments as described above. These results are summarized in figure 3 showing a curve which clearly indicates that there is a fairly wide region in the spectrum from 505 to 590 $\mu\mu$ or from light blue to yellow, where the stimulating efficiency is equal to, or more than 50 per cent. of white light. The maximum of this region is in the light green from 530 to 545 $\mu\mu$ where the stimulating efficiency is between 90 and 94 per cent., or practically the equivalent of white light.

In the region of the spectrum from the yellow-orange through the red end, or from 590 to 700 $\mu\mu$, there is a very sharp drop in the effectiveness of light, until at 700 $\mu\mu$ this is less than 5 per cent.

of white light. On the other side of the maximum there is a fall in value from 480 to 440 $\mu\mu$, but this is not as sharp as on the red end. From 420 into the ultra-violet there is a flattening of the curve which indicates strongly that ultra-violet light has a distinct stimulating effect. This effect, however, is very limited in comparison with the maximum in the field of green.

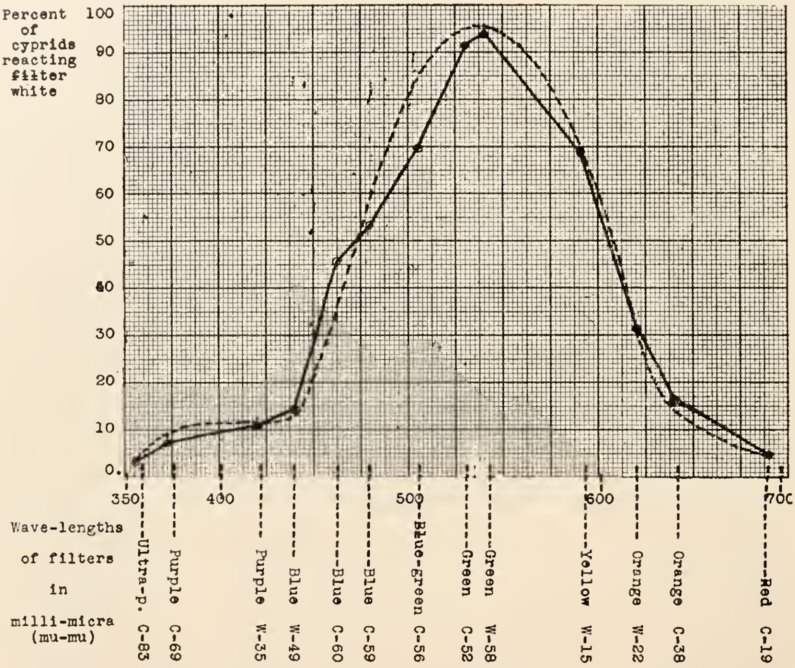

FIG. 3. A curve showing the distribution of the stimulating efficiency of equal energy values among the various parts of the spectrum, for the cyprid larvæ of *Balanus amphitrite* and *Balanus improvisus*.

Due to the fact that light from a tungsten filament does not penetrate far into the ultra-violet, we were unable to test this region as carefully as desired, but in all tests the relative energy values were carefully calculated.

No filter transmitting ultra-violet was available which did not at the same time transmit some light in the far-red end of the spectrum. (Compare No. 69 in Fig. 1.) The efficiency values for these filters were therefore corrected for the presence of red by

TABLE II.

RESULTS OF TWENTY SETS OF EXPERIMENTS TO DETERMINE RELATIVE EFFECTS OF EQUAL LIGHT ENERGIES IN DIFFERENT FIELDS OF THE SPECTRUM, UPON THE CYPRID LARVÆ OF BARNACLES.

Number of Filter	Ultra C 83		Purple C 69		Purple W 35		Blue W 49		Blue C 60		Blue C 59		Blue C 56		Green C 52		Green W 58		Yellow W 15		Orange W 22		Orange C 38		Red C 19	
Dominant Wave Length	355 μμ		370 μμ		420 μμ		440 μμ		460 μμ		480 μμ		505 μμ		530 μμ		540 μμ		590 μμ		620 μμ		640 μμ		690 μμ	
Limits of Transmission	315–428 μμ 690–Red End		310–485 μμ 690–Red End		300–475 μμ 650–700 μμ		400–510 μμ		335–640 μμ		335–690 μμ		340–700 μμ		425–670 μμ		485–635 μμ		500–700 μμ		545–700 μμ		540–Red End		620–Red End	
Number of Trial.	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter	White	Filter
1	27	3	25	3	28	2	30	3	20	7	28	13	19	10	14	13	14	15	13	11	26	12	24	6	31	4
2	28	2	27	5	26	2	25	5	14	10	30	10	19	12	19	16	20	15	24	14	20	9	28	11	32	4
3	33	1	28	1	28	2	30	4	19	7	18	12	16	11	21	19	18	15	19	15	19	7	29	7	34	1
4	30	3	30	3	32	2	24	3	18	10	21	11	19	12	17	16	19	14	23	16	18	8	23	3	33	2
5	26	1	24	2	26	4	23	2	14	9	13	10	14	10	18	17	19	15	21	13	26	7	26	6	32	2
6	29	1	27	2	27	4	24	3	14	6	16	7	15	11	13	10	19	15	23	15	25	6	24	4	27	2
7	22	2	27	1	27	4	28	5	23	9	23	10	22	14	16	22	16	15	22	18	25	6	33	6	35	1
8	36	2	32	3	26	3	32	4	24	9	20	10	13	11	12	15	17	16	20	13	29	5	24	3	26	1
9	20	1	18	2	28	3	22	4	18	6	17	14	15	13	15	16	19	17	19	11	26	5	30	5	33	1
10	32	2	33	4	25	3	29	4	20	14	18	12	16	12	14	15	17	14	19	14	24	6	22	2	32	1
11	32	1	30	1	25	4	32	5	19	10	18	14	21	15	21	19	11	15	17	9	20	7	24	3	25	2
12	35	1	32	3	22	4	33	2	22	9	24	12	21	15	20	15	16	10	20	11	24	7	26	2	30	1
13	29	2	28	2	27	4	28	3	23	8	22	10	21	15	18	15	18	14	17	13	23	8	25	5	30	0
14	30	1	27	6	29	3	32	3	20	14	15	8	20	10	22	17	17	19	15	10	22	6	29	3	29	1
15	36	0	32	2	25	5	32	3	20	8	20	7	18	15	18	18	15	15	22	15	18	6	29	4	37	1
16	34	1	31	2	25	4	30	5	23	13	21	12	24	17	21	17	12	11	20	14	22	7	24	3	29	2
17	34	1	33	1	26	4	28	4	30	5	24	8	26	10	20	18	12	14	19	14	21	4	30	4	31	1
18	35	2	27	4	26	2	27	6	20	11	21	13	19	14	20	17	13	19	21	13	21	8	27	3	31	0
19	25	1	32	2	23	4	31	4	25	13	23	16	25	16	20	17	12	20	14	12	24	8	27	5	30	1
20	30	0	29	2	27	3	26	3	25	10	28	15	18	12	18	15	13	12	19	15	20	9	33	3	35	0
Total number of reactants to either light	603	28	572	51	528	66	534	75	411	188	420	224	381	265	357	327	319	300	387	266	453	141	537	88	622	28
Per cent. of stimulating efficiency as determined by dividing the number reacting to white with the number reacting to filter	4.6% Corrected for Red 3.2%		8.9% Corrected for Red = 7.4%		12.5% Corrected for Red = 10.9%		14.2%		45.8%		53.4%		69.6%		91.6%		94.0%		68.7%		31.1%		16.4%		4.5%	

determining the percentage of red transmitted and then deducting that value from the total value on the basis of the effectiveness of that region in the red.

During the course of this work on cyprids it was also found possible to test the reactions of the nauplii of four species of barnacles to these lights as described above. These results were uniform in that the region of the spectrum between 530 and 545 $\mu\mu$ was in each case the most stimulative. This maximum stimulating effect was the same both when these organisms were negative and when positive to light.

It is of interest that our results are in substantial agreement with the work of other investigators using nauplii of barnacles: Loeb & Maxwell, 1910, Loeb & Westeneys, 1916 and Loeb & Northrup, 1917. Thus it is apparent that the same region of the spectrum offers the greatest stimulating efficiency in both the nauplius and in the cyprid which is not surprising in view of the fact that the nauplius eye is still present in the cyprid.

CONCLUSIONS.

The cyprid larvæ of barnacles agree with the larvæ of most other marine organisms in that they react to light. With equal radiant energies and with the infra-red heat rays eliminated, a distinct difference in stimulating effectiveness is found between different regions of the spectrum. This difference shows, for the cyprids of the two species of barnacles tested, a region of maximum efficiency in the light green, between 530 and 545 $\mu\mu$. It is of interest to note that similar results have been found for the larvæ of other marine organism. The larvæ of the hydroid, *Eudendrium* are most sentitive to blue light (460–480 $\mu\mu$); the larvæ of *Arenicola,* to the green (about 495 $\mu\mu$); and the larvæ of the squid. to blue green (470–510 $\mu\mu$).

However from the results obtained by Hess (1910), Mast (1917) and Hecht (1921), it is apparent that the distribution of stimulating efficiency in the spectrum is not necessarily the same for all organisms, which react to light. It is consequently apparent that there is no true color vision in these lower forms—in the usual interpretation of the term, but rather that the photo-sensitive materials in the light perceiving organs are more sensitive to the effects of certain wave-lengths than to others. Increased intensity of other wave lengths may however produce similar results.

This investigation has, however, an important bearing on the problem of the nature and extent of the fouling of ships' bottoms, an intensive study of which is being completed by the senior author and of which this work forms a part. It has been found that light is an important factor governing the attachment of the organisms which cause fouling and that at the time of attachment many of these larvæ are negative to light, for they are found most abundantly on the shaded portions of the ships' hull.

Since barnacles are the most important factor in fouling it seems probable that the studies reported above may have some significance in the solution of this problem, for if a light green paint were used in place of the red, now universally used, a considerable benefit should be derived, on theoretical grounds, provided all other factors were comparable.

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BIOLOGICAL BULLETIN

VITALITY OF THE GAMETES OF *CUMINGIA TELLINOIDES.*

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From the Marine Biological Laboratory, Woods Hole, Mass.

Cumingia tellinoides is a small lamellibranch mollusk found abundantly in the Woods Hole region. Its breeding season extends over a period of three or three and a half months, beginning early in June. The production of eggs is continuous and spawning by each mature individual occurs two or three times during the course of the breeding season. Eggs may be had in great abundance during this time. One female may shed 300,000 eggs, although the average number extruded is estimated to be between 100,000 and 200,000. They are expelled into the sea water where they are fertilized.

The present paper is a report on experiments to determine the vitality of the gametes, and more especially the longevity of the unfertilized eggs. It may be assumed that when eggs are released from the ovary, they are normal. From the time of liberation, deterioration begins and if not fertilized they ultimately go to pieces by fragmenting in a characteristic manner. The extensive work of Goldforb indicates that some deterioration takes place in sea urchin eggs while stored in the ducts of the gonad before extrusion. This may also be true of *Cumingia,* since two clear cases of physiologically poor lots of eggs have been found among hundreds of normal ones. Deterioration o both gametes of *Cumingia* and of *Arbacia* is rapid after extrusion into sea water.

VITALITY OF THE EGGS.

The eggs used in these experiments were obtained by isolating sexually mature individuals in small stender dishes filled with sea water into which the eggs were shed. The eggs, so collected in one dish, may therefore be conveniently referred to in the description of the experiments as a "batch" or a "lot" of eggs. The reference in all cases is to the eggs of a single female.

During the summer of 1926 many batches of eggs were kept without fertilization to test their longevity. During the progress of the experiments a few eggs were taken from the dishes from hour to hour and fertilized as a test of their physiological condition and of the rate of deterioration as indicated by decreasing percentage of fertilization. The experiments reported here deal principally with the longevity of the unfertilized eggs as indicated by the time of disintegration.

Approximately two per cent. of the eggs of most females are defective at spawning and fragment within four or five hours. The other ninety-eight per cent. remain intact and capable of development for longer periods. The poorest lot of eggs when tested at a temperature of 20° to 22° centigrade went to pieces completely in six hours, and it was not uncommon for 98 per cent of the eggs of some females to fragment in from eight to nine hours. The great majority of the lots of eggs fragmented in from nine to fifteen hours, the average being between ten and twelve hours. When tested at 18.5° to 20° C., on the other hand, the most vigorous batches of eggs, remained intact from fifteen to twenty hours, that is, the eggs retained their normal appearance for that length of time. It was shown that a majority of the eggs either refuse to develop or develop abnormally, when fertilized shortly before fragmentation. However, a few normal embryos come from the oldest eggs, even when the percentage of fertilization is greatly reduced. When a lot of eggs is fertilized after fifty per cent. of them have fragmented, it not infrequently happens that most of those which remain intact fertilize and cleave. However, most of them die in cleavage or gastrula stages and few develop into normal embryos. The oldest lots of eggs to develop normally were ten and twelve hours old and rarely fifteen hours old. These all showed 95 to 98 per cent. fertilization and normal development.

The extreme limits of longevity of the unfertilized eggs are therefore found to be six to twenty-six hours, and the limits of fertilization and normal development in the best lots of eggs are ten to fifteen hours. These figures are for temperatures normally experienced by the eggs of the species, or 18.5° to 22° C. They therefore represent the normal longevity of the eggs.

The statement by Morgan that the eggs of *Cumingia* will not stand rough handling is misleading. They are injured by centrifuging but there is abundant evidence that they develop normally under ordinary laboratory manipulation.

Tables I., II. and III. deal with various phases of the longevity and vitality of the eggs and include representative experimental data.

Individual Variation.

After any particular lot of eggs begins to fragment, three or four hours elapse before all or 98 per cent. have fragmented. The variation in longevity within a single group is, therefore, approximately four hours. For example, if 10 per cent. of the eggs have fragmented at twelve hours, the average expectancy of fragmentation for the remainder would be 30 per cent. in thirteen hours, 50 per cent. in fourteen, 70 per cent. in fifteen, and 95 to 98 per cent. in sixteen hours. Two per cent. may remain intact indefinitely. The rate of fragmentation is shown in Table I., as is also an actual comparison of the longevity of five lots of eggs from five females studied under identical conditions. Tables II. and III. show the same thing in slightly different form.

When fresh eggs are fertilized by fresh sperm the percentage of cleavage is usually between 97 and 100 per cent. However, a few lots of eggs gave from 89 to 95 per cent. cleavage. One lot of physiologically poor eggs was observed which when fertilized with physiologically good sperm gave only 20 to 30 per cent. cleavage. This sperm when used to fertilize other lots of eggs gave from 97 to 100 per cent. cleavage showing that failure to cleave on the part of these eggs was not due to defective sperm. This lot of physiologically poor eggs was slimy from the first and showed a tendency to cling to the containing dish. This together with one other lot of defective eggs may be regarded as confirmation of Goldforb's contention that eggs at the time of spawning may show all the symptoms characteristic of aging

TABLE I.

VITALITY OF *Cumingia* EGGS.

Record of five lots of eggs spawned by five females on August 16 and kept unfertilized at 20° C. The vertical columns, when read from top to bottom, give the result of eleven examinations that were made with the microscope at approximately hourly intervals in order to ascertain the time that elapsed before fragmentation began in each lot of eggs as well as the rate of fragmentation. The percentages refer to the number of eggs that remain normal in appearance. From three to five hundred eggs were counted in each examination.

	♀ No. 1.	♀ No. 2.	♀ No. 3.	♀ No. 4.	♀ No. 5.
After 5½ hrs.	100% normal	97% normal	100% normal	100% normal	100% normal
After 6½ hrs.	70% normal	100% normal	100% normal	100% normal	100% normal
After 8 hrs.	20% normal	5% normal	100% normal	100% normal	100% normal
After 9 hrs.	0% normal	0% normal	100% normal	100% normal	98% normal
After 10 hrs.			100% normal	100% normal	98% normal
After 11½ hrs.			100% normal	98% normal	90% normal
After 12 hrs.			100% normal	98% normal	80% normal
After 13 hrs.			98% normal	95% normal	50% normal
After 14 hrs.			95% normal	85% normal	30% normal
After 14½ hrs.			90% normal	70% normal	2% normal
After 15 hrs.			80% normal	50% normal	0% normal

Comment.—Eggs from lots 3, 4 and 5 were inseminated with freshly shed sperm after 11½ hours. The percentage fertilization which resulted was estimated to be 90 to 95 per cent. and abundant normal larvæ developed. Many eggs are apparently normal for 12 hours after extrusion. Eggs from lot 3 were fertilized 13 hours after extrusion which gave 75 per cent. cleavage and normal development. It appears that there is great variation in the vitality of the eggs of different females. These five lots spawned by five different females during the same hour were treated alike. One fragmented in seven hours, one in eight hours, one in fourteen hours, one in sixteen hours and it may be predicted in the light of former experience that the other would have fragmented completely in seventeen or eighteen hours if the experiment had been continued. No. 1 began to fragment in six hours. This gives the range of variation in longevity among the eggs from the five females as six to eighteen hours and normal fertilization for twelve hours in the best of the five. These five lots of eggs are substantially typical and may be considered as representative, although some are considerably longer lived than those used in this experiment. At least twenty such experiments were carried out during the season. The extreme limits of longevity were found to be from five to twenty-six hours. Only one or two per cent. of the best lots of eggs remained intact after the expiration of that time. It is interesting to compare this with the egg of *Arbacia* which, according to Goldforb and others, has a longevity of 40 to 48 hours.

TABLE II.

NORMAL VARIATION IN PERCENTAGE CLEAVAGE IN THE EGGS OF *Cumingia tellinoides.*
Temperature 22° C.

August 4. Freshly spawned eggs from ten females were fertilized by the sperm of one male to show the initial variation in the percentage of cleavage of eggs of this species. This table also shows the effect of aging. It is interesting to note that after 90 to 95 per cent. of the eggs have fragmented those remaining intact may fertilize and cleave. (Compare Tables II. and III.) All eggs normal and fragmented, are included in the calculation of the percentages.

	After 2 Hrs.	After 8 Hrs.	After 11 Hrs.	After 14 Hrs.	After 16 Hrs.
Female No. 1........	98% cleavage	98% cleavage	93% cleavage	10% cleavage	0% cleavage
Female No. 2........	98% cleavage	98% cleavage	40% cleavage	0% cleavage	0% cleavage
Female No. 3........	92% cleavage	10% cleavage	0% cleavage	0% cleavage	0% cleavage
Female No. 4........	97% cleavage	70% cleavage	71% cleavage	50% cleavage	2% cleavage
Female No. 5........	98% cleavage	7% cleavage	0% cleavage	0% cleavage	0% cleavage
Female No. 6........	96% cleavage	97% cleavage	92% cleavage	60% cleavage	20% cleavage
Female No. 7........	99% cleavage	98% cleavage	96% cleavage	2% cleavage	0% cleavage
Female No. 8........	95% cleavage	95% cleavage	95% cleavage	40% cleavage	10% cleavage
Female No. 9........	88 to 90% cleavage	30% cleavage	20% cleavage	1% cleavage	0% cleavage
Female No. 10........	30% cleavage	0% cleavage	0% cleavage	0% cleavage	0% cleavage

eggs. The first vertical column of Table II. shows the usual range of variation in freshly spawned eggs in respect to percentage fertilization and cleavage.

TABLE III.

LONGEVITY OF THE EGGS OF *Cumingia tellinoides*, TEMPERATURE 22° C.

The eggs of the ten females compared in Table II. in respect to percentage fertilization are here compared as to longevity and time of fragmentation or disintegration. It will be noted that the time of reduction in cleavage is almost synchronous with the time of fragmentation. This relationship does not always hold because in numerous experiments the percentage of cleavage fell off before fragmentation. In other words, if the reduction in cleavage due to aging and the rate of fragmentation were plotted as curves they would not as a rule be superimposed or parallel.

	Percentage of Fragmentation with Aging.			
	After 7 Hrs.	After 11 Hrs.	After 15 Hrs.	After 16 Hrs.
Female No. 1.........	0%	0%	50%	100%
Female No. 2.........	0%	45%	100%	100%
Female No. 3.........	75%	100%	100%	100%
Female No. 4.........	0%	2%	72%	90%
Female No. 5.........	73%	100%	100%	100%
Female No. 6.........	0%	0%	40%	50%
Female No. 7.........	0%	0%	99%	100%
Female No. 8.........	0%	0%	12%	60%
Female No. 9.........	0%	7%	100%	100%
Female No. 10.........	100%	100%	100%	100%

Note.—Many of the eggs of lots 4, 6 and 8 lived beyond sixteen hours. The other lots of eggs had fragmented completely after sixteen hours.

VITALITY OF THE SPERMATOZOA.

The work of Gemmill ('00) and F. R. Lillie ('15) has shown that variations in the concentration of sperm suspensions of *Arbacia* make a great difference in the duration of their fertilizing ability. The writer accordingly used several sperm dilutions in order to learn whether the same phenomena are exhibited by the sperm of *Cumingia.*

Spermatozoa shed by a mature male *Cumingia* of average size in 30 cc. of sea water was estimated by actual measurement to be a 2 to 3 per cent. suspension.[1]

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[1] The method of measuring the percentage was to kill the spermatozoa by the addition of formalin and after settling they were measured en masse. From this the calculation of the concentration of the original suspension was a simple matter.

This concentrated suspension, though at best somewhat variable, was used as a standard suspension from which various dilutions were made. It was found possible to select suspensions of approximately the same strength and this is a matter of more importance than that the exact percentage be known.

Two drops[2] of standard sperm suspension when added to 25 cc. of sea water was estimated to be a 1/500 to 1/750 per cent. suspension; two drops in 50 cc. of sea water was considered to be a 1/1000 to 1/1500 per cent. suspension; one drop in 50 cc. of sea water makes a 1/2000 to 1/3000 per cent. suspension, etc. The last named suspension when fresh is adequate to fertilize one hundred per cent. of the eggs whereas greater dilutions sometimes gave only partial fertilization. What was estimated to be a 1/4000 to 1/6000 per cent. suspension gave from eighty to one hundred per cent. fertilization and usually one hundred per cent. All of these suspensions were used and also the same percentage suspensions in larger quantities of sea water.

The method of studying the relative longevity of these various dilute sperm suspensions was to make up, by the proper dilutions, several dishes of each from freshly shed sperm. To these fresh eggs were added in turn at hourly intervals until the suspensions no longer gave fertilizations. It was shown in general that the weakest suspensions die first. Tables 4 and 5 show that the longevity of the spermatozoa depends largely upon the degree of concentration. Even a suspension of 1/500 per cent. shows some preserving effect in that spermatozoa live for a longer time in this concentration than in greater dilutions. There is little difference between a 1/2000 and a 1/6000 per cent. suspension, and these no doubt represent natural conditions so far as longevity is concerned.

In work reported at this time the writer had in mind to study the longevity of gametes under natural conditions. In general the longevity of the sperm in the most dilute suspensions is somewhat less than that of the eggs. In a few cases using suspensions of 1/500 per cent. approximately ninety per cent. normal embryos developed from sperm and eggs that were twelve hours old and in numerous other cases at nine and ten hours. As a rule when sperm suspensions of 1/2000 to 1/3000 per cent. are used very few fertilizations occur after nine or ten hours. Numerous experiments show that spermatozoa begin to die after three and one half or four hours. The indication therefore is that a majority of the spermatozoa under natural conditions live from four to nine hours.

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[2] The same pipette was used in making all dilutions of an experiment and one giving approximately one cc. per 20 drops

TABLE IV.

LONGEVITY OF THE SPERM OF *Cumingia* IN VARIOUS DILUTIONS AS SHOWN BY THE PERCENTAGE OF FERTILIZATIONS THAT THEY GIVE WITH FRESH EGGS (JULY 25).

Age of the Suspension Tested.	1 Drop in 100 Cc. Sea Water 1/4000 to 1/6000%.	1 Drop in 50 Cc. Sea Water 1/2000 to 1/3000%.	4 Drops in 50 Cc. Sea Water 1/500 to 1/750%.	8 Drops in 50 Cc. Sea Water 1/250 to 1/500%.
2 hours................	99%+	99%+	100%	100%
$3\frac{1}{2}$ hours..............	96%	95%	97%	96%
7 hours................	17%	24%	99%	97%
10 hours...............	2%	8%	63%	72%

For this experiment the spermatozoa used were all from the same male. The eggs were from three females and were not over three hours old when used. Controls showed them to be practically 100 per cent. normal eggs.

TABLE V.

LONGEVITY OF THE SPERM OF *Cumingia* IN VARIOUS DILUTIONS TESTED ON JULY 23.

Sperm all from one male.

Age of Sperm Suspension Used.	1 Drop in 50 Cc. Sea Water 1/2000 to 1/3000%.	2 Drops in 50 Cc. Sea Water 1/1000 to 1/1500%.	4 Drops in 50 Cc. Sea Water 1/500 to 1/750%.	6 Drops in 50 Cc. Sea Water 1/300 to 1/500%.
$2\frac{1}{4}$ hours...	84% cleaved	100% cleaved	100% cleaved	99%+ cleaved
$4\frac{1}{6}$ hours...	35% cleaved	65% cleaved	75% cleaved	80% cleaved
$7\frac{1}{2}$ hours...	15–20% cleaved	30% cleaved	50–60% cleaved	85–87% cleaved
9 hours....	0% cleaved	0% cleaved	2% cleaved	35–40% cleaved

This table shows that sperm in 1/2000 to 1/3000 per cent. suspension died in nine hours. Other experiments have shown that from ten to forty per cent. of the spermatozoa in these most dilute suspensions often survive for 10 to 12 hours. Table IV. is more typical in this respect, but even in this case the percentage of fertilization is below the average. The point that is shown clearly is that spermatozoa die first in the most dilute suspensions and that a suspension of 1/500 to 1/750 per cent. suspension preserves the life of the sperm beyond their normal life in the open sea.

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When the work of Gemmill and Lillie is taken into consideration it is apparent that Goldforb's experiments on the aging of spermatozoa do not represent normal conditions. He apparently relied upon concentrated dry sperm which are known to age much more slowly than sperm kept under natural conditions. The aging of sperm in concentrated suspensions has no particular significance. His work on the eggs is certainly not open to the same criticism but I should question his interpretation and results on the aging of spermatozoa. The work of the other two authors and this present work show that the life of spermatozoa in dilute suspensions is brief.

The statement by Lillie that the spermatozoa of *Arbacia* lose ability to fertilize eggs in a few minutes in dilute suspensions is not verified for *Cumingia* sperm, although the theory that they gradually lose something to the water which is essential to fertilization and which ultimately renders them incapable of bringing about the fertilization reaction may very well be true.

It has been my observation that spermatozoa that are able to swim actively are capable of fertilizing eggs; and that they do not lose the ability to initiate the fertilization reaction after a few minutes as reported by Lillie for *Arbacia*. Up to the present time no attempt has been made to learn whether or not the spermatozoa of *Cumingia* contain an activating substance, essential to fertilization. In many ways the spermatozoa of *Cumingia* behave as those of *Nereis* and *Arbacia* do, but if they contain a sperm receptor (Lillie, 15) preliminary experiments indicate that it is dissipated in the sea water more slowly than in the cases investigated by Lillie. Experiments on this question are in progress, as well as investigations of the cause of excessive polyspermy. These experiments are designed for comparison of the gametes of *Cumingia*, *Chætopleura* and *Hydroides* with the work of Goldforb and Gemmill on sea urchin eggs and with Lillie's work on the spermatozoa of *Nereis* and *Arbacia*.

Activation of Spermatozoa.

Spermatozoa, after having apparently lost their vitality, may be revived. When placed in a dish with eggs they become activated and swim vigorously. The activation of sperm in the presence of eggs was observed repeatedly. The stimulus from the eggs or egg water is evident almost instantly, but not all of the spermatozoa are so activated. I interpret this to mean that most of the quiescent spermatozoa are already dead or weakened beyond the possibility of functioning.

The phenomenon of activation of apparently spent sperm by the exudations from eggs is interesting and not uncommon. The physiological value of this activation is evident although the real cause is obscure. Lillie (13) shows that the spermatozoa of *Nereis* and *Arbacia* are positively chemotropic to weak acids and to egg secretions and are apparently stimulated by them.

Concentrated Sperm Suspensions.

It was learned that spermatozoa in concentrated suspensions retain their vitality for very long periods. Under such conditions they may swim from twenty-four to thirty-six hours and give 90 to 100 per cent. fertilization and normal development. They retain sufficient life for four days to show occasional contractions visible under a compound microscope. It is evident therefore that there is some protective element in this unnatural concentration. Cohn claims that it is carbon dioxide or hydrogen ion concentration. Dry sperm of fishes has the same extended longevity although possibly from different causes, not having had the initial stimulus to swim and use up its limited store of energy.

Discussion.

Goldforb expresses the belief that deterioration of eggs begins at the time of maturation while they are still stored in the gonads. He attributes the great variability of sea urchin eggs to the differences in time that they remain in storage before spawning takes place. I find a similar variation in the vitality of eggs of *Cumingia*, but it is noteworthy that maturation does not take place in the eggs of this species until after the entrance of the spermatozoön which occurs after extrusion into the sea water. Deterioration in the gonad in this case, therefore, could not be due to maturation. The fact that eggs vary so much in their longevity leads to the belief that some deterioration takes place before extrusion and before maturation. It is hardly likely that the great difference in vitality that experiment reveals is due entirely to natural variability. The eggs are stored in the gonads and their ducts for some time before extrusion and those that are stored longest may very well show physiological deterioration. Two cases of eggs which showed all the symptoms of aging at spawning verify Goldforb's contention on this point. At the present time I am unable to state whether the considerable variability in the longevity of *Cumingia* eggs is due principally to normal variability or is in part due to physiological deterioration while in storage. Cases of low percentage fertilization in the eggs of *Cumingia* are rare. As a rule they give 97 to 100 per cent. cleavage, although the longevity may be more variable.

SUMMARY.

I. The average longevity of *Cumingia* eggs when kept at a temperature of 20° to 22° C. is 10 to 12 hours as judged by time of fragmentation and ability to give a high percentage of normal embryos. The average longevity at 18.5° to 20° C. is 12 to 15 hours.

II. Approximately two per cent. of any lot of eggs may be defective as shown by their early fragmentation which occurs long before the rest.

III. The outstanding fact is the wide range of variation in the vitality and longevity of *Cumingia* eggs. The eggs of a single individual vary by four hours or approximately 25 per cent., while the eggs of different females vary in their longevity from six to twenty-six hours or over 400 per cent.

IV. Eggs in rare instances show deterioration at spawning, apparently due to long-time storage in the ducts of the gonads. Most lots of eggs when freshly spawned give from 97 to 100 per cent. cleavage and normal development.

V. The longevity of the best sperm in suspensions of 1/400 to 1/500 per cent. is 10 to 12 hours as judged by functional activity and ability to give 90 to 100 per cent. fertilization and normal development. In sperm suspensions of 1/2000 to 1/3000 per cent. it is 4 to 7 hours, but from 30 to 50 per cent. fertilization may frequently be expected from suspensions 7 to 12 hours old and 1 to 5 per cent. fertilization from suspensions 12 to 20 hours old.

VI. Spermatozoa in concentrated suspensions of 1 to 3 per cent. retain their vigor for many hours. They frequently give 90 to 100 per cent. fertilization after twenty-four to thirty-six hours, and some individual spermatozoa live for four days, still showing faint contractions of the tail at intervals. This preservation of the life of the spermatozoa is attributed to the presence in the water of CO_2 produced by the activity of the spermatozoa, or to hydrogen ion concentration.

VII. Spermatozoa after becoming quiescent show activation in the presence of eggs or egg water.

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CONDITIONS DETERMINING THE ORIGIN AND BEHAVIOR OF CENTRAL BODIES IN CYTASTERS OF *ECHINARACHNIUS* EGGS.[1]

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INTRODUCTION.

Statement of the Problem Concerning the Origin of Central Bodies.

In every cell generation there are differentiated the various cell components such as chromosomes, plastids, Golgi bodies, chondriosomes, central bodies, secretory droplets and similar elements. In the case of certain ones, such as chromosomes, the synthetic processes producing new units occur only by the growth and division of preëxisting bodies of the same kind. Such a component has genetic continuity from cell to cell as an individualized body, appearing to play some rôle in its own formation. Its origin may therefore be termed autogenic or self-perpetuating. In contrast to this, the synthetic processes forming other components, such as secretory droplets, are not localized about preformed structures of the same kind. Such a component has no genetic continuity as an individualized body and its origin is commonly described as occurring de novo.

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[1] The experiments were performed at the Mt. Desert Island Biological Station, Maine, 1923, and at the Marine Biological Laboratory, Woods Hole, Massachusetts, 1926.

Central bodies[2] have special interest with reference to this problem of protoplasmic differentiation, since in some cases they arise by the growth and division of preëxistent central bodies, while in other instances they apparently are formed de novo. In some cells they persist from one cell division to another, maintaining their identity as individualized structures during the vegetative period when the mitotic mechanism, of which they appear to be the formative foci, has disappeared. At a certain time in the cell cycle they divide and the daughter cells receive central bodies that have genetic continuity with that of the mother cell. For example, in spermatogenesis, the central body of the sperm can frequently be traced as a self-perpetuating individualized structure to the central body of the primary spermatocyte.

On the other hand there is also much evidence for a de novo origin of central bodies. In higher plants where centers are absent, blepharoplasts which have the characteristics of central bodies appear de novo in the final divisions of the germ cells. In animal fertilization it has yet to be proved that the central body of the sperm aster has genetic continuity as an individualized structure with the central body of the sperm, except in a few forms such as *Cerebratulus* (Yatsu, '09), and in most cases its formation may be de novo. When eggs are artificially activated in such a way that cytasters are formed, a study of sectioned material shows that they have central bodies (Morgan, '96, '99, '00; Wilson, '01; Hindle, '10; Herlant, '19; Fry, '25*b*; Tharaldsen, '26; etc.). Since cytasters appear simultaneously in large numbers and not by division the central bodies are apparently formed de novo.[3]

---

[2] The term central body is used throughout this paper in its broad non-committal sense, denoting any differentiated structure present at the central region of an aster, exclusive of the inner ends of the rays (and the possible presence of chromatin). The term involves no implications as to whether or not the central body is composed of a granule, the centriole, and a surrounding zone, the centrosome, either of which may be present or absent in various organisms, and both of which may show many modifications (Wilson, '25, pp. 30 and 673).

[3] For a detailed discussion of the literature on this subject see Wilson "The Cell in Development and Inheritance," 1925, especially Chapter IX.

The idea that all central bodies are self-perpetuating structures received an impetus due to the early theory of fertilization developed by Boveri and Van Beneden. They assumed that the chief event in fertilization is the introduction by the sperm into the egg of a preformed division center and hence that the central body of the sperm aster has genetic continuity as an individualized structure with that of the sperm (Boveri, '01). So generally accepted was this assumption that when artificial parthenogenesis was discovered, where the aster appears about the egg's nucleus, it was taken for granted that the egg nucleus must therefore have such a preformed central body that is ordinarily quiescent under the normal conditions of fertilization. To explain cytasters according to this theory it was necessary either to regard them as "pseudoasters," assuming that if they do not arise about preformed centers they are not "true asters," or to assume that central bodies lie scattered through the cytoplasm as a result of hypothetical rapid divisions of the egg's hypothetical division center (Meves, '02; Wassilieff, '02; Petrunkewitsch, 04). These ideas which are without proof are only of interest to illustrate the strength of the underlying assumptions which are still generally accepted, *i.e.*, that central bodies have genetic continuity as individualized structures, and that asters ordinarily arise about such units on the theory that the central body is the persistent element of the astral mechanism acting as the formative focus.

A possible explanation of the seemingly conflicting evidence that in some cases central bodies have an autogenic origin and in others a de novo one has been proposed by Wilson. He suggests that central bodies which seem to arise de novo, may actually be self-perpetuating structures that are submicroscopic during most of their history; they grow to a visible size only at certain times when they give the impression of having a de novo origin. This suggestion is in harmony with the general assumption that central bodies are individualized structures maintaining genetic continuity and giving rise to asters. He states: ". . . the cytologist is led on to the conclusion that the ultramicroscopical dispersed particles of the hyaloplasm may be as highly diversified chemically as are the visible formed bodies;

that they may be of all orders of magnitude; and that it is they which constitute the sources, or at least the formative foci, of those larger formed bodies that we have so often, but erroneously, assumed to arise de novo. For my part, I am disposed to take a final step by accepting the probability that many of these particles (I do not say all) as if they were ultra-microscopical plastids, may have a persistent identity, perpetuating themselves by growth and multiplication without loss of their specific individual type" ('23, p. 28). "Could we accept such a view we could more readily meet some puzzling difficulties such, for example, as the apparent contradiction between the origin of a centriole de novo, and its origin by division of a preëxisting body of the same kind" ('25, p. 721).

Morgan ('96, '99, '00) has made the most careful study of the relationship between central bodies and the structure of the surrounding cytasters. As a result of his observations he raised certain questions, in sharp contrast to the generally accepted ideas, that have since been largely ignored: "Is this central deeply staining mass a distinct body, or only the innermost fused ends of the rays; or is it only a product of the reagent?" ('99, p. 477). The present study is an attempt to resolve these conflicting hypotheses concerning the origin and behavior of central bodies in cytasters.

Types of Evidence Valid for Proving a Self-perpetuating or a de novo Origin of a Cell Component.

The behavior of a cell component in hybrids yields the only conclusive evidence concerning the nature of its origin. For example, in hybrids the paternal chromosomes and their constituent genes continue to perpetuate themselves and produce their effects in the protoplasm of another species. This conclusively proves that they are autogenic, playing an important rôle in their own synthesis. It demonstrates that under normal conditions, within the protoplasm of their own species, they are not passively produced by more active elements, otherwise they would not continue to grow and divide in an alien protoplasm. There is similar evidence for the autogenic origin of certain plastids. When species with different plastids are crossed, the paternal type continues to perpetuate itself in a cytoplasm that normally contains a different kind of plastid. The autogenic origin of other cell components has not been subjected to this conclusive proof of their capacity to perpetuate themselves by growth and division in the protoplasm of another species. Such a study of central bodies, in reciprocal crosses between *Arbacia* and *Echinarachnius*, is now in progress, a report of which will be presented in a later paper.

After reviewing the facts concerning certain plastids, Morgan ('26) states: "It may not appear far fetched to assume that there may be other bodies in the cytoplasm that grow and divide and, by extension, it might not seem too extravagant to assume that protoplasm itself (except for its secretion products) consists of units that grow and divide and are inherited." He calls attention, however, to the fact, very significant in this connection, that when two species are crossed reciprocally they produce similar hybrids. If some of the cytoplasmic components autonomously perpetuate themselves, maintaining their characteristic form, then in reciprocal hybrids the adults should show cytoplasmic differences. In one cross the cytoplasm with any self-determining units is derived from one species, and in the opposite cross it comes from the other. Since the cytoplasm of the adults is similar in both, despite its differing sources in the two crosses, this shows that its components generally are not autogenic in such a manner as to maintain their identity. It indicates that the synthetic processes are under the control of the nucleus producing similar results in both cases. It is generally to be expected, therefore, with some exceptions such as certain plastids, that cytoplasmic components do not perpetuate themselves autogenically maintaining their characteristic identity.

A component's power of growth and division, therefore, within the protoplasm of another species is primary evidence for its autogenic origin; its power of growth and division in the protoplasm of its own species constitutes a second grade of evidence indicating such an origin. Were there no genetic evidence from hybrids as to the self-perpetuating nature of chromosomes and certain plastids and were their behavior studied only in the cells in which they normally occur, the fact that they produce new units by the growth and division of similar preëxisting ones indicates an autogenic origin. When centrioles of secondary spermatocytes are produced by the growth and division of centrioles of primary spermatocytes, this is evidence that at least they localize the synthetic processes forming new centriole substance and therefore are autogenic to that extent. In so far as there is evidence for the formation of chondriosomes, Golgi bodies and vacuoles from similar preëxisting units, to that extent is there probability of their autogenic origin.[3]

Wilson ('01) describes the division of central bodies in dividing cytasters of artificially activated eggs of *Toxopneustes*, noting the significance of this with reference to the self-perpetuating nature of such central bodies. Fry ('25*b*) studied the behavior of cytasters in nucleated and enucleated *Echinarachnius* eggs in both living and fixed material, and found division only in those instances when they secondarily established connection with chromatin. Tharaldsen ('26) found the same situation in cytasters of *Asterias*. Although a cytaster may divide, this has no significance concerning the origin of its central body unless it is conclusively proved: (1) that the central body is an individualized structure independent of the rays, and (2) that its division initiates, and is not just a result of, the division of the surrounding cytaster. Since both of these points are unproved, and since the present study brings strong evidence against the former, the facts concerning the division of central bodies in dividing cytasters are in too uncertain a state to use them as evidence of an autogenic origin.

The foregoing types of evidence concerning a component's self-perpetuating nature, based upon its power of division, whether within the protoplasm of another species or within its own, assumes that it is microscopically visible throughout its history. In those components where new units do not visibly arise by division, but apparently are formed de novo, evidence concerning their origin becomes more difficult to secure. As previously noted, they actually may be self-perpetuating bodies that are submicroscopic during part of their history, becoming visible only at certain times. "Manifestly it is quite illogical to affirm an origin de novo of any formed body because it first becomes visible at a particular enlargement, even the greatest at our present command" (Wilson, '23, p. 24). Data can be obtained, however which may strongly support either an autogenic origin or a de novo one. If a component is obviously the product of another component; if its behavior is invariably correlated with certain changes in surrounding structures, then the presumption is very strong that it is produced by them and has a de novo origin. If, on the other hand, it is obviously not the product of surrounding structures but rather is their formative and controlling factor; if it passes through a certain definite cycle which is to some degree independent of modifications in surrounding structures; such facts, depending upon the details of the evidence, may indicate its self-perpetuating nature. Data of this type were secured in the present study.

Experiments.

This investigation deals with the origin and behavior of central bodies in cytasters of artificially activated eggs of *Echinarachnius parma*, to ascertain whether they are de novo structures produced by the cytasters, or whether they are individualized structures having an identity independent of the surrounding cytaster which forms about them, and have further characteristics indicating a self-perpetuating origin. The experiments are arranged as follows: (1) keeping the environmental factors of activation constant, to observe the various effects of different fixatives upon rays and central bodies; (2) keeping the fixative constant, to observe the various effects of modifications of environmental factors such as temperature and osmotic pressure upon rays and central bodies; (3) keeping both fixative and environmental factors constant, to observe the effects of various intervals during the astral history upon rays and central bodies.

The Effects of Various Fixatives upon Rays and Central Bodies of Cytasters (*Chart I.*).

The eggs for this group of experiments, as well as the others to be reported later, were obtained by removing the oral surface of the animals and securing eggs from those individuals where they appeared upon the exposed ovaries. The activation techni-

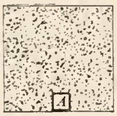
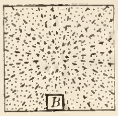
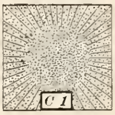
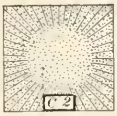
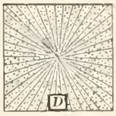
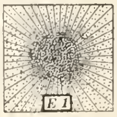
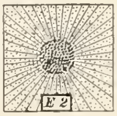
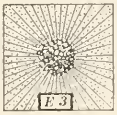
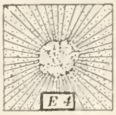
EXPERIMENT NUMBER	FIXATIVE		PERCENTAGES OF		
	NAME [THOSE CONTAINING ACETIC ACID ARE MARKED BY AN ASTERISK]	FORMULA	NO RAYS NO CENTRAL BODY	VAGUE RAYS NO CENTRAL BODY	DISTINCT RAYS NOT EXTENDING TO CENTER NO CENTRAL BODY [CENTRAL AREA STAINED SAME AS RAY AREA]
					
1	* FLEMMING-STRONG	1% Chrome acid 15 pts 2% Osmic acid 4 pts Acetic acid 1 pt			
2	FLEMMING-STRONG WITHOUT ACETIC	1% Chromic acid 15 pts 2% Osmic acid 4 pts		70	
3	* FLEMMING-WEAK	Strong Flemming 1 pt Water 1 pt		12	17
4	FLEMMING-WEAK WITHOUT ACETIC	Strong Flemming without Acetic acid 1 pt Water 1 pt		75	
5	OSMIC ACID	.025% Osmic acid		100	
6	CORROSIVE SUBLIMATE COLD - ALKALINE	Saturated solution - $HgCl_2$ - Cold - Made alkaline with NaOH		100	
7	CORROSIVE SUBLIMATE COLD - ACID	Saturated solution $HgCl_2$ - Cold - Has acid reaction	50	50	
8	CORROSIVE SUBLIMATE HOT - ACID	Saturated Solution $HgCl_2$ Heated to 90°C Has acid reaction	100		
9	* SUBLIMATE ACETIC 1%	Sat Solution $HgCl_2$ - 99 pts Acetic acid 1 pt	15	25	2
10	* SUBLIMATE ACETIC 5%	Sat. Solution $HgCl_2$ - 95 pts Acetic acid 5 pts	38	33	
11	* SUBLIMATE ACETIC 10%	Sat Solution $HgCl_2$ 90 pts Acetic acid 10 pts	15	50	2
12	* PICRO-ACETIC-STRONG	Sat sol Picric acid 1 pt Water 1 pt Acetic acid 5%		30	25
13	* PICRO-ACETIC-WEAK	Picro-Acetic-Strong 1 pt Water 1 pt		20	15
14	* BOUIN	Sat sol Picric acid 75 pts Formol 25 pts Acetic acid 5 pts		10	10
15	FORMOL 5% ALKALINE	5% Formol made alkaline with $MgCO_3$		100	
16	FORMOL 5% ACID	5% Formol Has acid reaction	14	86	
17	ALCOHOL 100%	100% Alcohol	25	75	
18	ALCOHOL 35%	35% Alcohol		85	15
19	* ZENKER	Potassium dichromate 2 gm Sodium sulphate 1 gm Water 95 cc Mercuric chloride 5 gm Acetic acid 5 cc		80	
20	ZENKER WITHOUT ACETIC	Same as above without Acetic acid		100	
21	* CARNOY and LEBRUN	100% Alcohol 1 pt Acetic acid 1 pt Chloroform 1 pt Mercuric chloride to saturation		5	20
22	CARNOY and LEBRUN WITHOUT ACETIC	Same as above without Acetic acid	2	45	15
23	* PETRUNKEWITSCH	Water 300 pts 100% Alcohol 200 pts Acetic acid 90 pts Nitric acid 10 pts Mercuric chloride to saturation		22	
24	PETRUNKEWITSCH WITHOUT ACETIC AND NITRIC	Same as above without Acetic and Nitric acids		94	2

CHART I. THE EFFECTS OF VARIOUS FIXATIVES

Activation: same in all experiments; a slight over exposure of the optimum butyric-hypertonic technique, cf. footnote 4, p. 372. *Fixatives:* as listed. *Times of fixation:* at frequent intervals after activation, cf. footnote 5, p. 372. *Percentages of types of cytasters:* averages of detailed counts made at the intervals at which eggs were fixed, from twenty-five minutes until one hour after activation; each count based on about 100 cytasters chosen at random from a number of eggs; examples of such data for Fixatives 3, 10 and 13, shown in Chart III, p. 380.

TYPES OF CYTASTERS AND CENTRAL BODIES

	DISTINCT RAYS NOT EXTENDING TO CENTER NO CENTRAL BODY [CENTRAL AREA STAINED LIGHTER THAN RAY AREA]  C 2	DISTINCT RAYS EXTENDING TO CENTER NO CENTRAL BODY  D	DISTINCT RAYS EXTENDING TO CENTER CENTRAL BODY VAGUELY DEMARKED GRANULAR  E 1	DISTINCT RAYS EXTENDING TO CENTER CENTRAL BODY CLEARLY DEMARKED GRANULAR  E 2	DISTINCT RAYS EXTENDING TO CENTER CENTRAL BODY CLEARLY DEMARKED CORPUSCULAR  E 3	DISTINCT RAYS EXTENDING TO CENTER CENTRAL BODY CLEARLY DEMARKED RETICULAR  E 4
*1		54		46		
2		30				
*3	12	41	11	2	3	2
4		25				
5						
6						
7						
8						
*9		30	27	1		
*10		17	12			
*11		31	1	1		
*12	20	23	2			
*13	9	20	6	29		1
*14	15	56	1	6	1	1
15						
16						
17						
18						
*19		20				
20						
*21	75					
22	2	36				
*23	55	23				
24	4					

UPON RAYS AND CENTRAL BODIES OF CYTASTERS.

Illustrations: show only the central regions, cf. Fig. 2, p. 390. *Result:* when eggs containing similar cytasters produced by similar activation are fixed by various fixatives, central bodies are present only when the fixative coagulates the cytaster in such a manner that rays are distinct and reach the center. For further details cf. p. 369. *Secondary result:* with few exceptions, only fixatives containing glacial acetic acid (indicated by an asterisk) clearly fix rays. For further details cf. p. 375.

que used was that developed by Just ('19). An egg sample of each individual was given a preliminary test of their capacity to form clear membranes by treating them with butyric acid solution (2 cc. 1/10 *N* butyric acid plus 50 cc. sea water). Only those eggs were used that gave over ninety per cent. membranes.

An artificial activation for *Echinarachnius* that is optimum for producing the maximum number of normal embryos yields only about ten per cent. eggs with cytasters (Fry, '25*a*). The activation used in the present experiments, therefore, was a slight over exposure so as to produce about twenty-five per cent. eggs with cytasters to facilitate their study.[4] The eggs show an average of seven per cent. cytolysis, although there is considerable variation as to this in the different fixatives.

Cytasters of *Echinarachnius* arise about fifteen minutes after removal from the activating solutions. None divide except occasional ones that secondarily establish contact with chromatin, and if they are numerous, cleavage does not usually occur (Fry, '25*b*). Thus the majority of cytasters formed soon after activation remain unchanged for hours except as to modifications of their detailed structure which are studied by fixing samples of eggs at various intervals.

Eggs were fixed in twenty-four fixatives being placed in each at frequent intervals after activation.[5] They were sectioned 5 μ thick and stained in Heidenhain's hæmotoxylin. Various degrees of destaining, carefully studied with water immersion

[4] Experiments 1 to 4 inc. and 7 to 14 inc., Chart I, were activated with a 35 second treatment with butyric acid solution (2 cc. 1/10 *N* butyric acid plus 50 cc. sea water), followed by a 20 minute interval in sea water, followed by an 18 minute treatment with hypertonic sea water (5 cc. 2.5 *N* sodium chloride solution plus 50 cc. sea water) at 24° C. Experiments 5 to 6 inc. and 15 to 24 inc. were similarly activated with butyric acid solution, followed by a similar interval in sea water. In treating them with hypertonic sea water (same concentration), however, the eggs were divided into four lots and exposed 15, 20, 25 and 30 minutes respectively. The experiment was so arranged that all lots came out of the hypertonic solution at the same time, and then they were mixed. The temperature was 22° C. That the two groups received an essentially similar activation is shown by the fact that samples of both contained about the same percentages of various types of development at twelve hours after activation.

[5] Experiments 1 to 4 inc. and 7 to 14 inc., Chart I., were fixed at the following intervals after activation: 15 min., 17.5, 20, 22.5, 25, 27.5, 30, 35, 40, 45, 50, 55, 1 hr., 1 hr.-15 min., 1–30, 1–45, 2 hrs., 3, 4, 5. Experiments 5 to 6 inc. and 15 to 24 inc. were fixed at the following intervals: 10 min., 15, 20, 30, 45, 1 hr., 2, 3.

lenses, show no significant differences in results. Stains other than hæmotoxylin were not used as it has been generally found that they yield similar results, and hæmotoxylin is the accepted standard.

The percentages of the various types of cytasters and their central bodies shown for each fixative, Chart I., are averages of a number of detailed counts, one made for each interval when eggs were fixed, from twenty-five minutes to one hour after activation (cf. footnote 5, p. 372). This is the period during which cytasters are at their maximum development. Each count for each interval is based upon the study (at 750 × magnification) of about 100 cytasters and their central bodies selected at random from a number of eggs. Such detailed counts for Experiments 3, 10 and 13, Chart I., are shown in Chart III., p. 380.

Since sections were cut at 5 μ, and since the majority of cytasters are 10 to 15 μ in diameter, most of them are found in two or more serial sections. In the case of each cytaster only that section was studied that includes its mid-region (Fig. 1, *b*, p. 384), ignoring the others (Fig. 1, *a* and *c*). The drawings show only the central portion of such mid-sections. The peripheral area is omitted to save space on the charts since it yields no significant information concerning rays and central bodies not shown by the central area. An entire mid-section of a cytaster is illustrated, Fig. 2, p. 390; the dotted line makes clear how small a portion of the central region is shown in the chart illustrations. Although the drawings are necessarily diagrammatic they are an accurate representation of the various classes of cytasters and their central bodies. They have the following classification (cf. Chart I.):

Rays.	*Central Body.*	*Chart Symbol.*
None	None	A
Vague	None	B
Distinct, not extending to center:		
Central area stained same as ray area	None	C1
Central area stained lighter than ray area	None	C2
Distinct, extending to center	None	D
Distinct, extending to center	Vaguely demarked—granular	E1
Distinct, extending to center	Clearly demarked—granular	E2
Distinct, extending to center	Clearly demarked—corpuscular	E3
Distinct, extending to center	Clearly demarked—reticular	E4

When cytasters that are in a similar condition due to similar activation, are fixed with a variety of fixatives, central bodies are present only when the fixative coagulates the cytaster in such a manner that the rays are distinct and extend to the center. The fact that rays are distinct and reach the center does not guarantee the presence of central bodies, since many cytasters with such rays are without them (Type D). In the majority of such cases the rays are more delicate than in those cytasters having central bodies, where are usually found rays of maximum coarseness and clarity (Types E1, E2, E3 and E4). Central bodies are never present when rays are absent (Type A), or vague (Type B), or clear without reaching the center (Types C1 and C2). Differences in the size of cytasters, which may vary from 5 to 25 μ, do not affect this relationship between the presence of central bodies and distinct central rays, nor does the location in the egg which may be central or peripheral.

Within any one egg there are found only related types such as, A and B, or B and D, etc. Unrelated types do not occur together. There is a similar occurrence of related types among the different eggs of the same slide, fixed at a given interval after activation. Since the percentages of the different types shown for each fixative of Chart I. are averages of a number of detailed counts made at various intervals after activation, the variety of types found in the general average is usually greater than those found at any one interval.

Type A, without any rays, is easily identified as a cytaster since it is a granular area that stains darker than the surrounding cytoplasm, of the same size as a typical cytaster, and is frequently associated with those having vague rays. This rayless type is often found during the late history of asters when the rays frequently fade out completely.

In this series of experiments, concerning the effects of fixatives (Chart I.), the twenty-four lots of eggs which were placed in the twenty-four killing agents, at various intervals, had received a similar activation. Hence the living eggs, prior to fixation, contained about the same array of living cytasters. Therefore the wide diversity in cytaster structure found on the slides is the result of differing coagulation products of various fixatives which differently coagulate the same phenomena. It will be noted, for example, that Type C, with clear rays not extending to the center, is entirely absent in certain fixatives but present in others in considerable numbers. Again, some of the reagents show large percentages of Types D and E having clear central rays, whereas others show only Types A and B, which are either without rays or have only vague ones. The significance of the relation between coarse clearly-fixed central rays and the presence of central bodies will be discussed later (p. 383).

In those fixatives containing glacial acetic acid (marked on Chart I. with an asterisk) there is a total of sixty-nine per cent. cytasters with distinct rays (Types C1 to E4), and thirty-one per cent. with rays absent or vague (Types A and B). On the other hand, in those without glacial acetic acid there is a total of ten per cent. with distinct rays and ninety per cent. with no rays or vague ones. The possible significance of acetic acid with reference to the chemical composition of astral rays will be discussed in a later paper. Experiments have been carried out concerning the effects of acetic acid, related acids, and acetates, at various hydrogen ion concentrations, as well as similar studies of other fixing agents.

The Effects of Modifications of Environmental Factors upon Rays and Central Bodies of Cytasters (*Chart II.*).

In the preceding experiments the activation was constant and the fixation was varied; in the present series the fixative is constant and the environmental factors of activation are varied. Chart II. presents the various modifications of activation used, *i.e.*, treatment with both weak and strong hypertonic sea water, each for forty, sixty and eighty minutes, to study the effects of differences in osmotic pressure; treatment with butyric acid solution followed by exposure to hypertonic sea water, for ten, twenty, and thirty minutes, to study the effects of modifications of the butyric-hypertonic activation technique; modifications of temperature, at two degree intervals between 20° C. and 28° C., the range within which cytasters occur when using an optimum activation, to study temperature effects; treatment with one per cent. and five per cent. ether, each for three, fifteen and

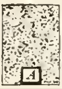
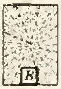
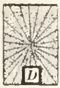
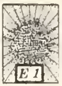
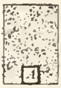
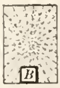
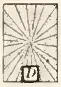
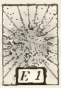
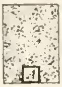
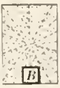
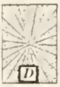
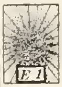
EXPERIMENT NAME AND NUMBER		ACTIVATION DATA						PERCENTAGE OF CYTASTER EGGS 1 hr. AFTER ACTIVATION	PERCENTAGE OF CYTOLIZED EGGS 1 hr. AFTER ACTIVATION
		ETHER		BUTYRIC ACID	HYPERTONIC SEA WATER		TEMPERATURE C.		
		PERCENTAGE OF ETHER ADDED TO SEA WATER	LENGTH OF TREATMENT WITH ETHER AND SEA WATER MIXTURE	LENGTH OF TREATMENT WITH 2cc $\frac{1}{10}$N BUTYRIC ACID PLUS 50cc SEA WATER [FOLLOWED BY 20 min INTERVAL IN SEA WATER BEFORE HYPERTONIC TREATMENT]	NUMBER OF cc OF 2.5 N NaCl ADDED TO 50cc SEA WATER WHEN MAKING HYPERTONIC SEA WATER	LENGTH OF TREATMENT WITH HYPERTONIC SEA WATER			
MODIFICATIONS OF WEAK HYPERTONIC TREATMENT	IA	No Ether Treatment		No Butyric Treatment	5c.c.	40 min.	17	5	0
	IB	"	"	"	"	60 min.	"	14	5
	IC	"	"	"	"	80 min.	"	16	10
MODIFICATIONS OF STRONG HYPERTONIC TREATMENT	IIA	No Ether Treatment		No Butyric Treatment	12c.c	40 min.	17	7	22
	IIB	"	"	"	"	60 min	"	28	55
	IIC	"	"	"	"	80 min	"	35	52
MODIFICATIONS OF BUTYRIC HYPERTONIC TREATMENT	IIIA	No Ether Treatment		35 sec.	5c.c.	10 min.	17	12	0
	IIIB	"	"	"	"	20 min	"	20	0
	IIIC	"	"	"	"	30 min	"	60	0
MODIFICATIONS OF TEMPERATURE	IVA	No Ether Treatment		35 sec.	5 c.c	17 min	20	0	0
	IVB	"	"	"	"	"	22	1	0
	IVC	"	"	"	"	"	24	45	0
	IVD	"	"	"	"	"	26	.1	33
	IVE	"	"	"	"	"	28	0	85
MODIFICATIONS OF 1% ETHER TREATMENT	VA	1%	3 min.	33 sec.	5c.c.	30 min	18	3	0
	VB	"	15 min	"	"	"	"	5	10
	VC	"	35 min.	"	"	"	"	6	14
MODIFICATIONS OF 5% ETHER TREATMENT	VIA	5%	3 min.	33 sec.	5 c.c.	30 min	18	10	30
	VIB	"	15 min	"	"	"	"	.1	40
	VIC	"	35 min.	"	"	"	"	0	83

CHART II. THE EFFECTS OF MODIFICATIONS OF ENVIRONMEN-

Activation: modified as listed. *Fixative:* all experiments fixed with saturated corrosive sublimate plus 2.5 per cent. glacial acetic acid. *Times of fixation:* as listed. *Percentages of types of cytasters:* each count for each experiment, at each of the three intervals of fixation, based on about 100 cytasters chosen at random from a number of eggs.

PERCENTAGES OF TYPES OF CYTASTERS AND CENTRAL BODIES

[FIXED WITH SUBLIMATE ACETIC 2.5%]

	1/2 HOUR AFTER ACTIVATION				1 HOUR AFTER ACTIVATION				2 HOURS AFTER ACTIVATION			
	NO RAYS NO CENT. BODY	VAGUE RAYS NO CENT. BODY	DISTINCT RAYS TO CENTER NO CENT. BODY	DISTINCT RAYS TO CENTER GRANULAR CENT BODY	NO RAYS NO CENT. BODY	VAGUE RAYS NO CENT. BODY	DISTINCT RAYS TO CENTER NO CENT. BODY	DISTINCT RAYS TO CENTER GRANULAR CENT. BODY	NO RAYS NO CENT. BODY	VAGUE RAYS NO CENT. BODY	DISTINCT RAYS TO CENTER NO CENT. BODY	DISTINCT RAYS TO CENTER GRANULAR CENT BODY
	 A	 B	 D	 E 1	 A	 B	 D	 E 1	 A	 B	 D	 E 1
I A		90	10			10	65	25		12	72	16
I B		80	20			13	74	13	[No Experiment]			
I C		31	46	23		50	29	21	19	50	19	12
II A	[No Experiment]					60	37	3	25	69		6
II B	[No Experiment]					19	60	21	90	10		
II C	[No Experiment]					7	73	20	86	14		
III A	No Cytasters					12	38	50		24	66	10
III B	No Cytasters					17	39	44	[No Experiment]			
III C		1	56	43		8	71	21	[No Experiment]			
IV A	No Cytasters				No Cytasters				No Cytasters			
IV B			34	66			75	25		50	50	
IV C		45	55			45	25	30		87	13	
IV D		40	60			22	78			80	20	
IV E	No Cytasters				No Cytasters				No Cytasters			
V A	17	63	15	5		20	60	20		14	26	60
V B			95	5		8	17	75		60	18	22
V C	11	33	11	45		27	21	52		24	46	30
VI A		10	15	75		60	30	10		15	45	40
VI B	20	60	20		50		20	30	29	29	40	2
VI C	No Cytasters				No Cytasters				No Cytasters			

TAL FACTORS UPON RAYS AND CENTRAL BODIES OF CYTASTERS.

Illustrations: show only the central regions, cf. Fig. 2, p. 390. *Result:* when a fixative is used capable of clearly fixing rays, central bodies are present only when the modifications of the environmental factors of activation produce well-formed cytasters with distinct rays reaching the center. For further details cf. p. 375.

thirty-five minutes, preceding a butyric-hypertonic activation, to study the effect of an anæsthetic. The chart has a column showing the percentage of eggs in each experiment containing cytasters at one hour after activation. Another column shows the percentage of cytolysis. There are wide variations in both.

The eggs of all these experiments were fixed in sublimate acetic 2.5 per cent., *i.e.*, saturated solution of corrosive sublimate 97.5 per cent. plus glacial acetic acid 2.5 per cent. They were fixed at a half hour, at one hour, and at two hours after activation. Sublimate acetic fixatives (Chart I., Fixatives 9, 10, and 11) produce four types of cytasters, with few exceptions: (1) with no rays and no central body (Type A); (2) with vague rays and no central body (Type B); (3) with distinct central rays and no central body (Type D); (4) with distinct central rays and a vaguely demarked granular central body (Type E1). The greater variety of central body structure seen in such fixatives as weak Flemming or Bouin (Chart I., Fixatives 3 and 14) are not found in fixation with sublimate acetic.

Eggs were sectioned and stained as described above (p. 372). The percentages of cytaster types shown for each experiment, at each of the three intervals after activation when eggs were fixed, are each based on a study of about 100 cytasters and their central bodies selected at random from a number of eggs. In certain experiments, however, where cytasters are found in only one per cent. of the eggs, or less (Chart II., Experiments IV. B, IV. D and VI. B), the number of cytasters studied was about twenty-five.

In this group of experiments (Chart II.) the living eggs of the various ones contained widely differing percentages of various types of living cytasters, due to the variety of astral phenomena produced by the wide modifications of activation. These differences are visible in living cytasters by the use of a high power water immersion objective. Poorly formed cytasters appear as homogeneous structureless vesicles. Although well-formed ones do not show the clean-cut thread-like rays seen in fixed material, they clearly do show a radiate configuration. These eggs with their differing cytasters were all placed in the same fixative (sublimate acetic 2.5 per cent.), in contrast to the preceding experiments (Chart I.) where eggs with similar cytasters were placed in different fixatives. The results of both groups of experiments confirm each other. *Using a sublimate acetic 2.5 per cent. fixation, central bodies are present only when the various modifications of activation produce well-formed cytasters with distinct rays extending to the center.* This relationship holds true whether eggs having cytasters number 0.1 per cent. (Chart II., Experiments IV. D, VI. B) or 60 per cent. (III. C); whether the cytasters arise early (III. C, etc.) or late (III. A, III. B); whether there is no cytolysis (IV. A, etc.) or 33 per cent. cytolysis (IV. D, etc.); whether the cytasters are small (5 μ) and numerous, or large (25 μ) and few; whether they are located in the egg centrally or peripherally.

The meaning of the differences in percentages of types of cytasters at one half hour, one hour, and two hours after activation, will be discussed in the next section.

The Effects of Various Intervals in the History of Cytasters upon Rays and Central Bodies (*Chart III.*).

In the first group of experiments environmental factors of activation are constant and the fixative is the variable; in the second group the environmental factors are the variable and the fixative is constant. In the present group both fixative and environmental factors are constant and the variable is different intervals of time during the history of the cytaster.

Chart III. presents the percentages of classes of cytasters found at frequent intervals from fifteen minutes to two hours after activation, in the case of three typical fixatives where central bodies occur, *i.e.*, weak Flemming, sublimate acetic 5 per cent., and weak picro-acetic. The counts for each interval are based on the study of about 100 cytasters and their central bodies chosen at random from a number of eggs. This is the same material from which the data for Chart I. were obtained. The sectioning and staining technique were previously described (p. 372).

It will be observed that rays first appear at about fifteen minutes after activation, remaining vague for some minutes. Clear rays are not usually found until twenty minutes after

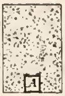
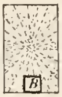
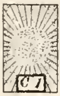
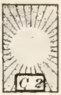
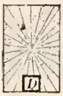
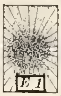
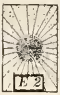
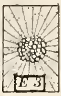
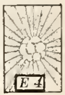
FIXATIVE	INTERVALS AFTER ACTIVATION WHEN COUNTS WERE MADE OF CYTASTERS AND CENTRAL BODIES	PERCENTAGES OF TYPES OF CYTASTERS AND CENTRAL BODIES								
		NO RAYS NO CENT. B'DY	VAGUE RAYS NO CENT B'DY	DISTINCT RAYS NOT EXTEND. TO CENTER NO CENT. B'DY CENTRAL AREA STAINED SAME AS RAY AREA	DISTINCT RAYS NOT EXTEND. TO CENTER NO CENT B'DY CENTRAL AREA STAINED LIGHTER THAN RAY AREA	DISTINCT RAYS EXTENDING TO CENTER NO CENT. B'DY	DISTINCT RAYS EXTENDING TO CENTER CENTRAL B'DY VAGUELY DEMARKED GRANULAR	DISTINCT RAYS EXTENDING TO CENTER CENTRAL B'DY CLEARLY DEMARKED GRANULAR	DISTINCT RAYS EXTENDING TO CENTER CENTRAL B'DY CLEARLY DEMARKED CORPUSCULAR	DISTINCT RAYS EXTENDING TO CENTER CENTRAL B'DY CLEARLY DEMARKED RETICULAR
		 A	 B	 C 1	 C 2	 D	 E 1	 E 2	 E 3	 E 4
FLEMMING WEAK	15 min.		100							
	17.5 "			100						
	20 "			25		55		20		
	22.5 "			20		80				
	25 "				50	50				
	27.5 "					100				
	30 "		45	10	35	10				
	35 "			10	15	35	30		10	
	40 "			28		28	29		10	5
	45 "			8		50	17		8	17
	50 "		40	30		20		10		
	55 "		12	47		40		1		
	1 hr.		5	25		35	25	10		
	1 hr.-15 min			50		50				
	1 " -30 "			34	16	34	16			
	1 " -45 "		17	16	17	50				
	2 hrs.		66			34				
SUBLIMATE ACETIC 5%	15 min	34	66							
	17.5 "	70	30							
	20 "	50	50							
	22.5 "	33				34	33			
	25 "	2	10			68	20			
	27.5 "		33			34	33			
	30 "		33			34	33			
	35 "		90				10			
	40 "	45	45			5	5			
	45 "	50	50							
	50 "	100								
	55 "	100								
	1 hr.	[No Experiment]								
	1 hr.-15 min	60	40							
	1 " -30 "	55	45							
	1 " -45 "	70	30							
	2 hrs	50	50							
PICRO-ACETIC WEAK	15 min	[No Experiment]								
	17.5 "	100								
	20 "	[No Experiment]								
	22.5 "	25		30		45				
	25 "		14		44	28	14			
	27.5 "			5		95				
	30 "			20	25	5	5	45		
	35 "		25	15	13		5	40		2
	40 "		50				16	34		
	45 "							100		
	50 "		12	31		19		38		
	55 "		40	30		20	10			
	1 hr.		40	30		20	10			
	1 hr.-15 min		40	20		20		20		
	1 " -30 "		13	25	6	50			6	
	1 " -45 "		23	10		35	12	12	4	4
	2 hrs		50	50						

CHART III. THE EFFECTS OF VARIOUS INTERVALS IN THE HISTORY OF CYTASTERS UPON RAYS AND CENTRAL BODIES.

Activation: same in all experiments; a slight over exposure of the optimum butyric-hypertonic technique, cf. footnote 4, p. 372; material same as that reported on Chart I, pp. 370–371. *Fixatives:* as listed. *Times of fixation:* as listed. *Percentages of types of cytasters:* each count for each interval of fixation, based on activation. Central bodies do not appear until rays have become clear, and they are never present unless the rays are distinct and reach the center. An hour or two after activation there is a tendency for the asters to become vague. When the rays become indistinct the central bodies disappear. This confirms the foregoing conclusion. *Central bodies are present only when distinct rays extend to the center of the cytaster. Cytasters pass through a formative period of vague rays when central bodies never occur; only after the rays have become clear and reach the center do central bodies appear; if the rays fade in the later history of the aster the central bodies disappear.* Thus clearly correlated with the appearance and disappearance of distinct central rays is the appearance and disappearance of central bodies. This relationship is somewhat modified by the effects of various fixatives (Chart III.) as to the percentages of types, and as to the exact time schedule, but the story is essentially the same in all.

The three fixatives reported in Chart III. are capable of showing distinctly fixed rays. The vague rays of the early and later periods of the astral history are not due to poor fixation. They are caused by the actual condition of the living rays at the time of fixation, since the same fixatives show distinct rays at the mid-period of the astral cycle. The central body phenomena shown in these three fixatives are the same as all those fixatives of Chart I. where rays are clearly fixed. Where rays are not clearly fixed there are no central bodies.

Similar results are shown in the experiments where activation is modified, Chart II. The eggs were fixed with sublimate acetic 2.5 per cent., at one half hour, at one hour, and at two hours after activation. It will be noted that in most of the experiments the highest percentage of vague (or absent) rays usually occurs at a half hour and at two hours after activation,

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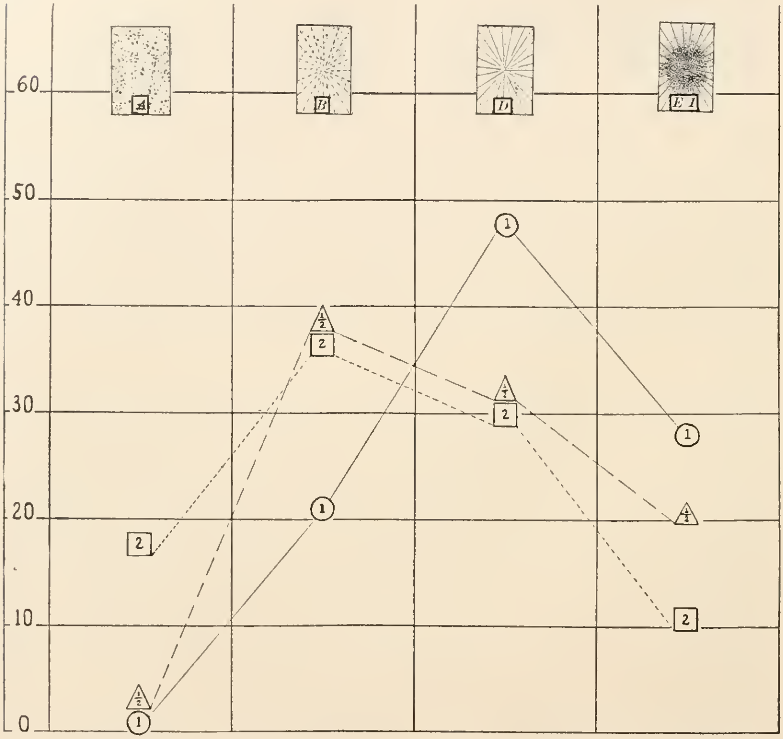
about 100 cytasters chosen at random from a number of eggs. *Illustrations:* show only the central regions, cf. Fig. 2, p. 390. *Result:* when an activation is used capable of producing well-formed cytasters, and a fixative is used capable of clearly fixing rays, if cytasters are studied at close intervals after activation, it is found that rays are at first vague and there are no central bodies; they appear only after rays have become clear and reach the center; they disappear when rays fade in the later history. For further details cf. p. 379.

whereas the peak of clear rays is usually at one hour. Since central bodies occur only when rays are clear and reach the center, they too show their optimum numbers at one hour, and are less numerous at one half and at two hours after activation. These relationships are shown in Graph 1. The percentages of



GRAPH 1. The effects of various intervals in the history of cytasters upon rays and central bodies.

This graph is derived from the data of Chart II., pp. 376–377. It shows the percentages of the various types of cytasters present at ½ hr., 1 hr., and 2 hrs., after activation, when all the detailed experiments concerning the effects of modified activation are averaged together. A triangle is the symbol for ½ hr. after activation, a circle for 1 hr., and a square for 2 hrs. Only cytaster types A, B, D and E1 occur, since the fixative used was sublimate acetic 2.5 per cent. The graph shows that asters with vague rays (or none) usually occur in maximum numbers at ½ hr. and at 2 hrs. after activation, whereas clear-rayed ones are most numerous at 1 hr. Also, the percentages of asters with central bodies follow the same sequence, indicating that the presence of central bodies is correlated with the presence of clear rays. For further details cf. p. 381.

each of the four types of cytasters (Types A, B, D and E1), at one half hour, one hour and two hours after activation, are in each case the total of all the detailed percentages of the various experiments for that type for that interval, shown on Chart II. A few of the individual experiments do not coincide with the average behavior, but such discrepancies are accounted for by the considerable differences in astral phenomena produced by the wide variations of activation. Despite wide modifications of osmotic pressure, temperature, butyric-hypertonic treatment, and ether treatment, producing different percentages of cytaster types, different percentages of eggs containing cytasters, different percentages of cytolyzed eggs, and different sizes and numbers of cytasters, the relationship between clear central rays and the presence of central bodies holds good.

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Discussion.

The Origin of Central Bodies in Cytasters of Echinarachnius parma.

The previously described experiments prove that when eggs have been similarly activated and therefore contain similar types and percentages of cytasters, if they are fixed with a variety of killing agents, central bodies are present only when the fixative coagulates the cytaster in such a manner that rays are distinctly fixed and extend to the center (Chart I.). When, on the other hand, a fixative capable of clearly fixing rays is kept constant, but the various environmental factors used in activation are modified, the same relationship holds good, and central bodies are present only when those environmental factors produce well-formed cytasters with clear central rays (Chart II.). Finally, when an optimum activation is used capable of producing well-formed cytasters, and they are fixed by a killing agent capable of fixing rays distinctly, if the cytasters are studied at close intervals after activation, it is found that they pass through an early vague-rayed condition when central bodies are never present; only after the rays become clear and reach the center do central bodies appear; and if the rays later fade, central bodies disappear. This is strong evidence that central bodies occur only if distinct rays reach the center of the cytaster (Chart III.).

Figure 1, *a*, *b*, and *c*, shows the appearance of three serial sections (each 5 μ) of a cytaster, Type E1, that was about 15 μ in diameter. Figure 1, *d*, is a schematic reconstruction of the entire aster. It is obvious that the top and bottom sections (Fig. 1, *a* and *c*) present a side view of rays in only the peripheral area, and that in their central regions the rays are seen in cross section as scattered dots. It is equally apparent that the middle section (Fig. 1, *b*) presents a side view of many of the rays throughout their entire length from the edge of the cytaster to its center, and that the center also contains the inner ends of those rays extending above and below which appear in cross section as closely crowded dots.

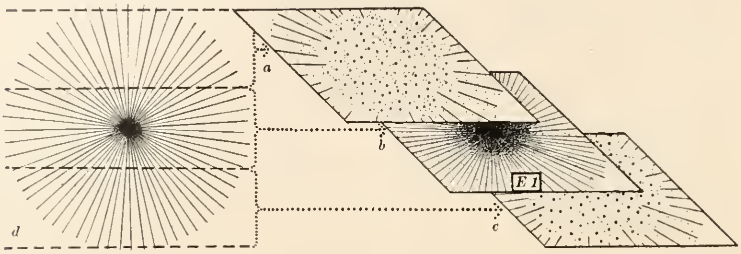

FIG. 1. The structural relations of rays and central bodies shown by a comparison of the serial sections of a cytaster with the reconstructed aster.

The serial sections (*a*, *b* and *c*), which were cut at 5 μ, are drawn from those of a cytaster having a vaguely demarked granular central body (Type E1). A schematic reconstruction of the entire aster (*d*) is shown at the left. In the top and bottom sections (*a* and *c*) rays are visible in side view in the peripheral area only, whereas in the central area they are seen in cross section as dots. In the middle section (*b*) many of the rays are visible in side view throughout their entire length, and the central portion also contains the closely crowded inner ends of the rays that extend above and below. These converge at the midpoint, and this when coagulated constitutes a central configuration, *i. e.*, the central body.

The following hypothesis is therefore proposed as the probable explanation of the so-called central bodies seen at the center of fixed cytasters. The rays converge at the mid-point of a cytaster. When they are coagulated by fixation their closely crowded inner ends are sufficient to explain a dark configuration at the center. Since cytasters differ as to number and coarseness of rays, there is a variety of coagulation products at the mid-points; they may be granular, corpuscular or reticular. *This study has conclusively proved that central bodies are de novo structures produced by the cytaster only after it has formed clear rays reaching the center. There is strong evidence for the further conclusion that these structures are produced only by coagulated cytasters and that they are nothing but the fixed closely crowded inner ends of well formed rays, and that they have no existence as individualized structures in the living cytaster.*

This explanation of central bodies in cytasters is supported by the fact that the type of cytaster with clear rays reaching to the center which nevertheless is without a central body (Type D), usually has rays that are more delicate than those of cytasters with central bodies (Types E1, E2, E3 and E4). Also if rays extend to the center but are vague, there are no central bodies (Type B). Therefore, only coarse well-formed rays form central bodies.

That central bodies are nothing but the coagulated bases of rays finds further support from the fact that not only must the rays be well formed, but they must also extend to the center. That type of cytaster with coarse distinct rays that do not reach the center (Type C) is of special significance in this connection. Were it not for this type it might be legitimately concluded that central bodies are individualized structures with a chemical nature similar to that of the rays; hence when rays are poorly and vaguely fixed, central bodies fail to be coagulated and appear to be absent; only when rays are well fixed are central bodies shown. The existence, therefore, of large numbers of cytasters with well-fixed distinct rays in the peripheral region that do not reach the center, disproves this possible interpretation since such cytasters never contain central bodies. If the assumption is correct that fixatives which fix central bodies are ones that fix rays, then central bodies should be present in this type of cytaster with well-fixed peripheral rays.

Almost conclusive evidence supporting the hypothesis that central bodies of cytasters are nothing but the coagulated inner ends of well-formed rays is found in the history of cytasters (Chart III.), where the appearance and disappearance of clear central rays is invariably accompanied by the appearance and disappearance of central bodies.

If, in the light of the above evidence, it is still claimed that central bodies of cytasters have an autonomous independent existence beyond the limits of microscopic vision, becoming visible only at certain times, then it must be assumed further that they always attain visibility simultaneously with the formation of distinct rays reaching the center, and again become submicroscopic whenever the rays fade; that they never attain visibility if clear rays are formed that fail to extend to the center or if vague rays arise that do reach the center. Were the central bodies of cytasters independent units having existence apart from the rays, their presence would not be so invariably dependent upon clear rays that reach the center. The wide fluctuations in structure and behavior of both central bodies and the surrounding rays caused by various fixatives, by modifications of environmental factors, and by different periods in the astral history, would not show the invariable correlation that they do. There would be at least some faint indications of central body behavior independent of clear rays reaching the center.

Although Morgan ('96, '99, '00) and Wilson ('01) did not reach such a conclusion, their numerous detailed observations of the behavior of central bodies and cytasters in *Sphærechinus*, *Arbacia* and *Toxopneustes* are the same as those of the present study. They note that cytasters are formed by the accumulation of a substance that only later assumes a radiate structure (Morgan, '96, p. 356; '99, p. 465, etc.; Wilson, '01, p. 558); that central bodies are not present at first and make their appearance only after the aster has passed through its early history (Morgan, '99, pp. 470 and 477; Wilson, '01, p. 558); that they are most apt to be present in well-formed cytasters (Morgan, '99, p. 477) whereas poorly developed ones do not contain central bodies (Wilson, '01, p. 560); that they are not present in all cytasters and show a considerable variety of structure (Morgan, '96, '99; Wilson, '01; numerous references); and that cytasters frequently fade out in their later history (Morgan, '99, p. 464; Wilson, '01, p. 554). As a result of these facts Morgan ('99, p. 477) tentatively proposed as a possibility, the conclusion established by the present study: "Whether the centers first form and the fibers (rays) arrange themselves around them, or whether the centers are the result of the focusing of the first formed rays at a central point is difficult to determine, for both centers and rays appear at about the same time. All things considered I am inclined to adopt the latter alternative."

The plates of Morgan ('96, '99) and Wilson ('01), as well as those of other investigators illustrating echinoderm cytasters (Herlant, '19; Tharaldsen, '26, etc.), show a diverse array of cytasters and central bodies, most of which are similar to those found in the present study of *Echinarachnius. Toxopneustes* (Wilson, '01), however, shows certain differences. Professor Wilson kindly permitted an examination of some of his slides, and it is clear that *Toxopneustes* cytasters contain a high percentage of a central body type somewhat like E2 of the present study, but it is more compact and very much smaller. In some cases it is granular; in others it is homogeneous and is identical in appearance with a typical period-like centriole (Wilson, '01, plates XV. and XVI.). Such differences, however, due to different species, are probably of no more significance that those caused by different fixatives or differing environmental conditions. Any variations in central body structure of cytasters, no matter what the cause, are meaningless, provided the conclusion of the present study is true, that such central bodies are nothing but various coagulation products of the inner ends of well-formed central rays, having no existence as individualized structures in the living cytaster. All previous investigators of echinoderm cytasters, from Morgan ('96) and Wilson ('01) to Fry ('25*b*) and Tharaldsen ('26), have assumed that the central bodies seen in fixed cytasters are structures having an actual existence in the living egg. The present study of *Echinarachnius* proves that in this species, at least, they are but the coagulated focal point of rays. It is probable that this is true of central bodies in cytasters generally, but proof of this awaits the application of a similar method of study to cytasters of other species.

The Method of Study and its Significance for Cytological Research.

If a cell component is studied in a fixed condition it is of course necessary to check such observations with a study of living material if this is possible. The study of living cells stained with vital dyes may yield information concerning the structure of a component when alive, to use as a basis of comparison for its appearance when coagulated. It is further necessary, if possible, to secure data concerning its chemical composition. Some components such as central bodies, are so small, however, that they cannot be seen in the living cell. Observations of such structures can be made only after the material has been coagulated and sectioned. When such is the case the greatest caution must be used in interpreting the results, keeping in mind the fact that a component when coagulated may present a very different appearance from the same component when alive.

The present study shows that various fixatives cause such a diversity of structure and behavior of central bodies, that to draw conclusions from observations made by the use of one or two killing agents might easily lead to erroneous results. A description of their structure based on fixation with Bouin's fluid would not harmonize with the results produced by sublimate acetic, and would be equally different from conclusions based on phenomena seen in material fixed with strong Flemming's fluid (Chart I.). Not only are the types and percentages of cytasters and their central bodies modified by the fixative, but the time schedule of events during the astral history is also changed. Clear rays and central bodies disappear at forty minutes after activation when eggs are fixed with sublimate acetic 5 per cent., whereas in weak Flemming's solution and in weak picro-acetic they are present until about an hour and a half after activation (Chart III.). Since the eggs were similarly activated, these time differences in the astral history are caused by the fixatives. The varying effects of different killing agents are also shown by the different percentages of cytolysis they cause, the data for which are not shown on Chart I. In the different sets, cytolysis varies from none to about fifteen per cent. at forty-five minutes after activation, and shows a much wider variation at later periods, depending upon the fixative used. Of all the factors of the environment capable of modifying the structure of a component, certainly no one of them can effect more radical changes than the killing agent. Hence if a component is studied only in a coagulated condition it is essential to know the modifications produced not only by one or two fixatives, but by a variety of typical ones. Only when these are known is there any hope of arriving at a conception of what is a "normal" condition, and even then it must be accepted with the greatest caution.

Not only is it important to know the effects of different fixatives, but it is equally important that in the case of each fixative, accurate counts be made of all the variations of a component produced, omitting none. All the coagulation products must be considered in arriving at a result and the percentages of each should be known. Had the method of study in the present investigation been one of random search, selecting certain types as "normal" and dismissing others as "artifacts," and had all the variations in cytaster and central body structure not been studied quantitatively, it is doubtful if the real relationship between clear central rays and the presence of the central body would have been apparent. Had that type with clear peripheral rays not reaching the center and having no central body (Type C), and the type with clear central rays without a central body (Type D), as well as vague rayed asters (Type B), been passed by as due to poor fixation, and had attention been centered only on those with central body structure, the real relationship between clear central rays and central bodies would probably not have been established. It cannot be said that any of the types are more or less important than the others, or that any are "normal" or "abnormal," in arriving at the conclusion.

That cytaster with clear peripheral rays not reaching the center, in which the central area is homogeneous and stained the same as the ray area (Type C1) occasionally contains a minute deeply staining granule at its mid-point (Fig. 2). This satisfies all the requirements of one of the a priori conceptions of a "normal" central body structure, *i.e.*, one having a period-like centriole, surrounded by a homogeneous zone, the centrosome, about which is the ray area. In fact, the present investigation began on this assumption, resulting in confusion due to the inability to explain the other types which were abundant in well-formed asters. That the central granule of this cytaster

(Fig. 2) is not a centriole, and that this aster is without significance for an understanding of central bodies in cytasters is shown by the following facts. (1) These granules are found only

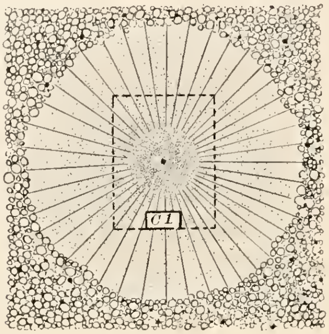

FIG. 2. Cytaster with distinct rays in the peripheral region only (type C1) containing a cytoplasmic granule at its midpoint.

This cytaster strikingly simulates a "typical" central body structure, one having a period-like centriole, surrounded by a structureless zone called the centrosome, about which is the ray area. Such a cytaster is without significance, however, concerning central body phenomena in cytasters. It occurs in but 0.5 per cent. of all cytasters. The granule is identical in appearance with those scattered through the cytoplasm. Two or three or more granules occur in such asters as frequently as does a single one.

The area within the dotted lines indicates that small portion of the midsections of cytasters shown in the chart illustrations, cf. p. 373.

in those cytasters where the rays do not reach the center (Type C1) and only in very few of them. In fact this cytaster with a centriole-like granule occurs in but 0.5 per cent. of all cytasters. Its relative scarcity was not appreciated until accurate counts were made of all the types. Until such a procedure is followed an observer can center attention upon a certain condition as typical and largely pass by other forms, and just because attention is centered on it, it may seem to be far more abundant than it actually is. Whether it occurs with a rarity that is significant can only be revealed by comparative counts. (2) The presence of a centriole-like granule in any one cytaster has no correlation with its presence or absence in other cytasters of the same egg. It is not a condition of central body structure difficult to fix, otherwise it would appear more frequently in the wide variety of fixatives used, and when present in one cytaster of an egg it would tend to be present also in others. (3) It is important to note that such granules occur in pairs and groups of three or more as frequently as they occur singly. Wilson ('01, p. 558) observed irregular numbers of centriole-like granules, especially in the early history of cytasters. He also notes (p. 560) that in well-formed ones they may be double. (4) Most important of all is the fact that they are identical in size and appearance with the darkly-stained granules that are abundant in the cytoplasm generally. Wilson ('01, p. 557) carefully notes that granules, sometimes occurring at the centers of cytasters, are similar to those scattered through the cytoplasm. In view of the preceding points, it is clear that in *Echinarachnius* cytasters such granules that simulate centrioles are actually cytoplasmic granules that happened to be at the center of an area where an aster formed; they remain there only if the differentiation of rays does not extend to the center (Type C); they are not found at the midpoint of cytasters with clear rays reaching the center (Type D) but these contain only the much larger central bodies of a very different character previously described and illustrated (Types E1 to E4). These centriole-like granules are crowded out of cytasters if the differentiation of clear rays extends to the center, by the same forces that eliminate all relatively large particles from between well-differentiated rays in asters generally.

Morgan ('99, p. 475) notes the effects of the several fixatives he used upon the variety of central body structure seen on the slides. As a result of this he raised doubts that have been confirmed by the present work: "How large a part does the reagent play? How much is artificial and how much actually represents what is actually present in the living egg? As to nucleus and chromosomes, all series show the same results. As to cytasters, and especially as to central bodies, the door is open to skepticism." *The present investigation is a further warning of the necessity of distrusting the coagulation products of certain cellular components such as central bodies, chondriosomes, Golgi bodies, etc.; of realizing that the phenomena seen on slides may be radically different from the living condition. On the other hand, it shows with equal clearness that results may be dependable, if the various typical coagulation products are known, if all the* *types produced are studied quantitatively, and if they are all taken into consideration in arriving at a conclusion.*

No profitable comparisons can be made between central bodies of cytasters and those of nuclear asters in *Echinarachnius* until the latter have been studied in a similar manner. The central bodies of the nuclear asters on the slides of the present experiments with artificially activated eggs, as well as those of cytasters that secondarily established connection with chromatin, have not been studied in detail, awaiting a controlled and quantitative study of central bodies in the nuclear asters of fertilized eggs. This is now in progress; a report of it will be presented in another paper together with a study of central bodies in fertilized eggs of *Arbacia* and *Asterias*.

Central bodies of nuclear asters in general show a great diversity of structure and behavior. In some forms they are described as autogenic, in others they appear to arise de novo; in some they seem to give rise to the aster, in others they appear only after the aster has been formed; in some they persist throughout the cell history, in others they disappear at certain phases of the cell cycle. In the light of the present study, this diversity of behavior, not to mention the variety of structure and function in the non-mitotic rôles of central bodies, makes it the more necessary to approach their future study in a controlled and quantitative manner. It is possible that if the present method is applied to the study of central bodies generally, that certain of the previous observations may be found to be based upon a single type arbitrarily selected as "normal" when using but one fixative, without knowing its quantitative relationships with respect to other types present on the same slides, and without knowing the differences caused by other fixatives. Whether or not such is the case awaits further study.

Résumé.

1. Some cellular components, such as chromosomes, arise by the growth and division of preëxisting bodies of the same kind, maintaining genetic continuity as individualized structures from cell to cell. They, therefore, have a self-perpetuating origin. In contrast to this, other components, such as secretory droplets,

have a de novo origin, since the synthetic processes forming new units occur without reference to preformed bodies of the same kind.

2. Central bodies (a term used in its broad sense without reference to various detailed modifications) are of special interest to this problem of protoplasmic differentiation, since there is evidence that in some cases their origin is de novo, while in others it is self-perpetuating.

3. Are central bodies of cytasters in artificially activated eggs of *Echinarachnius parma*, de novo structures produced by the aster, or are they self-perpetuating bodies, that are usually submicroscopic, becoming visible only at certain times and giving rise to the aster?

4. One series of experiments proves that when eggs have been similarly activated and therefore contain similar types and percentages of cytasters, if they are fixed with a variety (twenty-four) of killing agents, central bodies are present only when the fixative coagulates the cytaster in such a manner that rays are distinctly fixed and extend to the center.

5. A second series confirms the same result. When a single fixative capable of clearly fixing rays is used, but there are modifications of the various environmental factors of activation, such as temperature, osmotic pressure, etc., central bodies are present only when those environmental factors produce well-formed cytasters with clear central rays.

6. A third series also supports the preceding conclusion. When an optimum activation is used capable of producing well-formed cytasters, and they are fixed by a killing agent capable of showing distinct rays, if cytasters are studied at close intervals after activation, it is found that they pass through an early vague-rayed condition when central bodies are never present; only after the rays become clear and central do central bodies appear; and if the rays later fade, which frequently occurs, the central bodies disappear.

7. The cytaster, therefore, produces the central body only after it has formed clear rays reaching the center, no matter what are the modifications caused by fixatives, or environmental factors, or intervals of time in the astral history. Thus the

central body has a de novo origin. It is probable, moreover, that only a coagulated aster produces a central body and that it has no existence as an individualized body in the living cytaster. The mid-point of the aster contains the crowded converging inner ends of the rays. These when coagulated form the so-called central bodies seen at the centers of cytasters in fixed material. This hypothesis is supported by the following facts: central bodies are most abundant when rays are coarse, and are usually absent when rays are delicate, and always absent when rays are vague; there are many cytasters with coarse rays in the peripheral portion which do not reach the center, and these never contain central bodies; clearly correlated with the appearance and disappearance of clear central rays during the history of cytasters, is the appearance and disappearance of central bodies.

8. The investigation is another illustration of the need of a controlled and quantitative method in cytological study. When a cellular component is too small to be studied in the living condition, and must be studied only after coagulation, it is necessary not only to control the environmental factors, and to study it at frequent intervals during periods of significant change, but it is also necessary to know its various coagulation products formed by different typical fixatives. It is equally essential to know the exact percentages of all these variations, without exception, that they may all be taken into consideration quantitatively when arriving at a conclusion. To arbitrarily select a certain type as "normal" because it happens to coincide with a certain a priori idea, and to disregard the other types as "artifacts" produces uncertain results.

9. No profitable comparison of central body phenomena in cytasters of *Echinarachnius* can be made with those of its nuclear asters until the latter have been studied in a similar controlled and quantitative manner, a report of which will be presented in another paper.

10. Glacial acetic acid was present in some of the fixatives used and absent in others. Of all types of cytasters having clear rays (whether with or without central bodies), eighty-five per cent. are found in the presence of acetic acid. The possible

significance of acetic acid with reference to the chemical composition of astral rays is under further investigation.

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ACTION OF SALTS ON *FUNDULUS* EGG.

I. The Action of Na, K, and Ca Chlorides upon the Egg of *Fundulus*.

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Interest in the fundamental action of chemicals upon the egg, egg membrane, embryo, and larva of the marine fish *Fundulus* has centered largely around the work of Loeb and his associates (1–17). The results of this work seem to show that marked differences exist in reactions of the eggs and hatched embryos or larvæ to different salts. Marked degrees of salt antagonism are also pointed out. Inasmuch as the conclusions drawn from this work are based almost entirely upon the egg and hatched embryo or larva, it was thought highly desirable to recheck the observations, using eggs and embryos dissected out of the eggs, as well as hatched embryos or larvæ. By means of an operative technic devised by Doctor J. Nicholas[1] of Yale University, the details of which will be published elsewhere, it has been found possible to remove the outer egg membrane from the embryo and yolk sac without, in any apparent way, interfering with the further development of the embryo. As a matter of fact, embryos removed from the egg develop in sea water in as normal a way as those enclosed in the egg. It has thus been possible to compare the action of various salts directly upon the egg, the embryo dissected free from the egg membrane, and the larva hatched from the egg.

That Loeb conceived of a similarity in the action of salts upon the hatched embryo or larva and the embryo contained in the egg can easily be gathered from the following passages. In 1911 he says (6): ". . . if the fish is *out of the shell* the addition of $CaCl_2$ alone is no longer sufficient and the addition of KCl also becomes necessary." Other remarks in the same article seem also to indicate that the marked differences in the reactions to salts between the larva and embryo were not appreciated. In 1916 he says (8): "This prolongation of life through the addition of Ca is due not to an action upon the protoplasm but to a prevention of the diffusion of the NaCl into the egg, since if we take the embryo out of the egg (or use the newly hatched embryo) it is killed in 50 cc. 3 M NaCl + 1 cc. 10/8 M $CaCl_2$ inside of a few minutes." In his last paper on *Fundulus* (17) he says: "In 1905 the writer suggested as an explanation that a pure NaCl solution, if its concentration exceeded a certain limit, made the membrane of the egg more permeable, so that NaCl could diffuse into the egg, killing the embryo, while this increase in permeability was prevented by the presence of a low concentration of Ca." These and similar passages throughout his works seem to the writer to show that the marked differences in the reactions to salts between the embryo dissected out or freed from the egg membrane and the larva were not fully taken into consideration.

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[1] The writer is deeply indebted for the assistance rendered by Doctor J. Nicholas who was kind enough to carry out the operative technic involved in removing the egg membrane.

The present paper embodies results obtained from investigations carried out during the summer of 1927 at the Biological Laboratory, Cold Spring Harbor, on the effects of Na, K, and Ca chlorides on the eggs, embryos freed from the egg membrane, and larvæ of *Fundulus heteroclitus*.

METHODS.

The eggs were stripped directly from the female fish into finger bowls containing sea water and then fertilized. They were kept at room temperature (20°–25° C.) as well as at lower temperatures (15°–18° C.). Those at the lower temperatures naturally took longer to reach a given stage in development than those kept continuously at the higher temperatures. The eggs were usually washed for varying periods in distilled water before use in order to free them of adhering salts as suggested by Loeb (13–17). This procedure, however, seems to the writer necessary to but a limited degree, since similar reactions are given by eggs washed for varying periods except when the time factor is greatly lengthened, *i.e.*, to several days as was the case in many of Loeb's experiments (13–17).

The egg membrane was cut mid-laterally by means of fine pointed iridectomy scissors and the contained embryo gently rolled out of the shell by means of a fine probe. Eggs in as nearly as possible the same stages of development were used together with embryos dissected from similar eggs. It is of considerable importance that eggs and embryos be in the same stages of development, since marked age differences in reactions to some salts seem to exist. The length of exposure of the egg to water is also an important factor in changing the general consistency of the egg membrane. Both eggs and embryos were placed in covered Syracuse watch-glasses in 10 cc. of the solution and were constantly observed under a compound microscope during the course of the experiments. Five to ten organisms were used in a single watch-glass. It is of much importance to carry through experiments until the eggs hatch or the organisms die since in many cases the eggs live in certain solutions but upon further development and hatching the embryos quickly succumb. The embryos and eggs used ranged in developmental stages from those in which heart action was just beginning to those with fully developed circulation and ready to hatch. Newly hatched larvæ were used for comparison with the dissected-out embryos and eggs. The end-point observed and recorded in all the experiments herein reported was the time of cessation of the heart beat. Recovery of the heart beat in sea water was also noted but will be dealt with in a subsequent communication. The salts used in these experiments were c.p. NaCl, $CaCl_2$, and KCl made up in distilled water. Only the effects of normal solutions of these salts will be presented at this time since the results at this concentration are typical.

Results of Experiments.

Since in general the results of all experiments are qualitatively alike only typical experiments will be described.

N KCl.—KCl, as repeatedly pointed out by Loeb and his coworkers (1–17), acts with considerable rapidity on the egg, dissected-out embryo, and larva. In all, the heart quickly

Table I.

Salt.	Egg.	Embryo Freed from Membrane.	Larva.	Remarks.
N KCl	+ (75 min.)	++ (15 min.)	+++ (4–6 min.)	Penetrates membrane rather slowly
N $CaCl_2$	+ (98 min.)	+ (51 min.)	++ (30 min.)	Penetrates membrane rather slowly
N NaCl	young + (24 hrs.) older ○	○	+++ (10 min.)	Penetrates membrane rather slowly
Equal parts:				
N NaCl + *N* $CaCl_2$	○	+ (12–24 hrs.)	++ (22 min.)	No penetration
N KCl + *N* $CaCl_2$	++ (30 min.)	++ (20–25 min.)	++ (20 sec.)	Aids penetration
N NaCl + *N* KCl	+ (73 min.)	++ (38 min.)	+++ (15 min.)	Penetrates membrane

Explanation.—Shows general conception as to toxic action of salts upon eggs, embryos and larvæ. + = relatively slow toxic action; + + = a faster toxic action; +++ = a marked toxic action; ○ = non-toxic. Figures show average time for cessation of heart beat.

stops beating, the relative order of resistance being, larva $<$ embryo $<$ egg. The egg is about 4 times as resistant as the dissected-out embryo, and about 20 times as resistant as the larva. In the action of KCl upon the embryo within the egg, about three fourths of the time necessary to cause cessation of the heart beat is spent in the passing through the egg membrane (Table I.). Eggs with embryos in which the heart is just beginning to beat appear less resistant then older eggs.

N $CaCl_2$.—$CaCl_2$ is also extremely toxic for eggs, embryos, and larvæ, the relative order of resistance being larva $<$ embryo $<$ egg. The egg is about one half as resistant as the embryo, and about 3 times as resistant as the larva. Relatively less time is spent by the Ca in going through the egg membrane than by K, since the embryo outside of the egg is killed in less than one half of the total time required for cessation of the heart beat of the embryo within the egg membrane. No marked age differences in resistance to $CaCl_2$ seem to exist, since the organisms are killed at all embryonic stages in almost the same relative time.

N NaCl.—The action of NaCl is by far the most interesting of those studied, since the results obtained are quite different from those reported by Loeb (17). The relative resistance to NaCl is, larva $<$ embryo $<$ egg. This series, however, must be modified because with NaCl the question of age enters in a most amazing way. Freshly fertilized eggs, as pointed out by Loeb (2), are killed in solutions of NaCl. As the egg grows older its resistance to NaCl increases up to the time of hatching. Eggs in which the embryonic heart has just begun to beat are susceptible to NaCl, while the same embryo removed from the egg will usually live for days in the same solution. As a matter of fact, they live almost as long as it takes for the normal embryo in sea water to reach the time of hatching. The freshly hatched larvæ, however, are killed very quckly when put into the same NaCl solution (Table I.). A marked change in the resistance of the animal to NaCl thus takes place when the embryo is ready to emerge or emerges from the egg.

Equal parts $N\ KCl + N\ CaCl_2$.—In such a mixture the toxicity for the egg, embryo, and larva is about the same (Table I.).

The time taken to cause cessation of the heart beat, in the mixture however, is quite different from that required in the case of solutions of the individual salts, as is shown in Table I.

Equal parts N NaCl + N $CaCl_2$.—In this mixture the larva is killed in approximately 20 to 25 minutes. The embryo dissected from the egg membrane can survive from 6 to 24 hours while the eggs are not killed. As a matter of fact, eggs will hatch in the solution, and the hatched larva is quickly killed, showing quite conclusively that while in the egg the embryo is protected; once out, it quickly succumbs. The embryo dissected from the egg membrane is always found to be much more resistant than the larva (Table I.).

Equal parts N NaCl + N KCl.—Such a mixture is quite toxic for the egg, embryo, and larva, the relative resistance being: larva < embryo < egg. The egg is approximately 2 times as resistant as the embryo and about 5 times as resistant as the larva (Table I.).

Discussion.

The above results seem to the author to be of interest inasmuch as they definitely point out and show that any assumption as to the interior condition of the egg cannot be relied upon until satisfactorily tested and proven. Loeb (1–17), in most of his work on *Fundulus*, seems to have assumed that the resistance of the hatched embryo or larva was comparable to that of the embryo within the egg membrane. In the case of NaCl in particular this view is shown to be erroneous, and it is quite possible that further investigation will yield equally interesting results. That the egg membrane changes in consistency with age is quite apparent in the ease with which the membrane can be cut. Young fertilized eggs have membranes quite tough and turgid; eggs exposed to distilled water and at low temperatures for long periods of time have much softer and more pliable membranes. Associated with these structural changes are doubtless the marked physiological ones observed. Further proof of marked physiological changes in the egg membrane have come out of unpublished investigations recently conducted by Miss E. Yagle of this Laboratory, on the exosmosis of H_2O from the *Fundulus* egg of different ages. Loeb (6), also points out in several instances that such changes occur—*e.g.*, ". . . since the *newly fertilized* egg is killed more rapidly by a $m/2$ solution of NaCl than it is killed by the same solution one or two days after fertilization." Young eggs, therefore, are very susceptible to NaCl while older ones are quite resistant. Young embryos, on the other hand, are much less susceptible than are very old ones. The hatched embryo or larva, however, is extremely susceptible to NaCl. The question as to the fundamental action of NaCl upon the younger eggs with hearts just beginning to beat apparently is not one of a purely chemical nature but rather of an action on the membrane in which the embryo is doubtless killed by some secondary effects, possibly osmotic. In eggs thus killed the contained embryo always appears much shrunken in size.

By means of the dissection technic in liberating the embryos from the egg it is possible to study quantitatively the relative effects of the salt upon the membrane and contained embryo and also upon the embryo free from the egg membrane. KCl, for example, seems to spend about 75 per cent. of its total time effect upon the egg membrane and approximately 15–50 per cent. on the embryo. $CaCl_2$ on the other hand, spends about 60 per cent. on the membrane and 30–40 per cent. on the embryo. NaCl must exert most of its effect on the membrane in those cases in which it is toxic since for embryos removed from the egg it is relatively non-toxic.

The site of action of the three salts Na, K, and Ca chlorides has always been of considerable physiological interest. Loeb (17), in the case of *Fundulus*, seems to have attributed the fundamental action of these salts to the membrane—the process being purely a diffusion phenomenon. In young eggs NaCl perhaps does not penetrate the membrane while in older eggs the membrane seems freely permeable, since the contained embryo is not killed in the same solutions when removed from the egg. KCl and CaCl seem to penetrate the egg membrane, the Ca entering in a relatively slightly shorter time than the K. The Na + Ca antagonism as suggested by Loeb (2) must be a membrane phenomenon since the embryo removed from the egg or the hatched larva is quickly killed in such a mixture. Combinations of Na + K, however, act more like KCl alone and little if any antagonism seems to exist (Loeb, 1, 2). The combination K + Ca, on the other hand, seems to exert additive effects and to kill eggs and embryos in almost the same time.

Several additional facts noted in these experiments are of especial interest since they seem intimately concerned in any fundamental explanation of the salt effect upon the egg and embryo. The space between the egg membrane and embryo is at first very small, due to the large size of the yolk sac. As the embryo develops rather a large amount of fluid accumulates between the egg membrane and embryo. This increase in fluid perhaps has much to do in modifying the rates at which the embryo is killed while in the egg. Around the yolk sac and embryo there is also a delicate vitelline membrane, the properties of which seem of much importance in respect to the resistance of the embryo to various salts. If with a very fine pointed needle a minute hole is made in this membrane immediately ventral to the eye and the embryo, still normal, transferred to a solution of NaCl or left in a solution of NaCl which is not ordinarily toxic, it is quickly killed. It seems that this membrane is an important factor in determining the resistance of the embryo to NaCl. Further investigations are to be carried out on these points.

SUMMARY.

(1) By means of an operative technic it is possible to remove the egg membrane from the egg of *Fundulus heteroclitus* and to compare experimentally the action of salts on the egg, the embryo freed from the membrane and the newly hatched larva.

(2) The effects of normal solutions of K, Na, and Ca chlorides upon the above are reported.

(3) The embryo, freed from the egg membrane, is quite resistant to NaCl solutions while the hatched larva is quickly killed in the same solution.

(4) The resistance of the eggs to NaCl increases with age.

(5) K and Ca chlorides kill the dissected-out embryo much more quickly than the egg, while the recently hatched larva is much more sensitive to the two salts than is the embryo.

(6) Combinations of these salts show antagonistic action.

Na + Ca mixtures are not toxic for eggs but are markedly so for the embryo freed from the egg membrane and for the newly hatched larva.

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ON THE ABILITY OF CERTAIN MARINE INVERTEBRATES TO LIVE IN DILUTED SEA WATER.

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There are many reasons for believing that animal life originated in the ocean and has gradually spread through the ages into freshwater and land habitats (15, 21). In the past annelid worms invaded the soil, probably by a rather indirect route which led them first into freshwater and gradually out into the land (23). At the present time many animals in various parts of the earth show varying degrees of ability to live in diluted sea water (1, 3, 10, 11, 12, 21). Marine invertebrates which have been studied have an osmotic pressure in their blood which is approximately equal to that in sea water, but the mineral salts are somewhat less and the pressure is maintained by other substances, largely organic, which are present (1, 8, 9). The skins of various animals differ greatly in ability to control the exchange of chemicals between the body fluids and the surrounding medium. Adolph (1) studied the exchanges of substances through the skins of annelids and found that there was little resistance to them.

The writer felt that it would be of interest to determine the ability of representative marine annelids to live in diluted sea water, and during August 1927 made some observations in the Marine Biological Laboratory at Woods Hole. Thanks are due to Drs. J. A. Dawson and R. Bennitt who made suggestions and helped in the identification of several species. Mr. A. M. Hilton and his staff of collectors also made special efforts to secure materials.

Animals were brought in fresh from the field and placed as soon as possible in clean glass finger bowls containing 250 cc. of water. Tubicolous worms were removed from their tubes, except in the case of Hydroides, which was studied both in and out of tubes, and of Cystenides, which was left in its own tubes.

The water in all bowls was changed each morning, and oftener when it showed indications of becoming stagnant or when a worm bled. Dilutions were made with fresh water from the taps in the Marine Biological Laboratory. Sea water came from the same source. Page (13) has made a careful analysis of this water for mineral constituents.

The results of the observations are given in Table I. *Nereis virens*, *Laonice viridis*, and *Limulus polyphemus* showed a considerable degree of toleration for sea water diluted to one fourth its normal salinity. *Arabella opalina*, *Glycera dibranchiata*, and *Lepidonotus squamatus* lived for many days in one half sea water and one half fresh. Most of the worms tested lived several days in three fourths sea water. *Nereis* in higher concentrations of sea water climbed out of the dishes at intervals and was found in varying degrees of desiccation, hence some individuals probably died sooner than some of those in more dilute solutions. Every *Laonice* studied lived throughout the period of observation and was active at the end. The Hydroides in tubes did not live as long as those which were removed. This was probably due to the fouling of the water by small organisms in and on the mollusc shells to which the tubes of these worms were attached. The *Limuli* used were small individuals, less than 10 cm. long. All that were tested in solutions as low as one fourth sea water survived to the end of the observations. One individual lived 26 hours in a solution of one eighth sea water, and another lived two hours in fresh water.

Nereids have been observed in various localities to be noteworthy for their ability to endure considerable dilution of sea water (3, 7, 11, 13). In India there is a species of *Limulus* which lives in brackish water (3). Vaughn (24) found that several species of corals survived a reduction of twenty per cent. in the salinity of the sea water and he interpreted this as indicating that the ocean has been in the past less salty than now. The observations described in this paper show that many worms have similar toleration. The writer cannot see that general features of bodily structure and habitat are especially correlated with ability to survive in diluted sea water. Apparently delicate branchiate worms, like *Chætopterus*, *Diopatra*, and *Laonice*, endure fresh water about as well as apparently tougher worms, such as *Phascolosoma* and *Lumbricillus*. The only oligochæte observed, *Lumbricillus agilis*, was not as hardy as many polychætes when placed in diluted sea water. In low salt concentrations all the animals studied showed a tendency to swell and became turgid and extended. *Laonice* perhaps showed this least; *Lepidonotus* and *Lumbricillus* perhaps most; *Amphitrite* frequently bled and died soon. The individual *Nereis* which lived and was active for twenty-one days in a medium containing only one fourth sea water became very active and soon began to shrink to its normal size when replaced in undiluted sea water.

TABLE I.

TIME IN HOURS WHICH ANIMALS LIVED IN SEA WATER AND VARIOUS DILUTIONS OF IT.

A indicates that an animal was apparently in good condition when the observations were discontinued.

Name of Animal.	No. Observed.	Sea Water.				
		1	$\frac{3}{4}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Amphitrite ornata (Leidy)	12	65	115	7	2	
Arabella opalina (Verrill)	5		302*A*	302*A*	.7	
Arenicola marina (L.)	2				2	
Chætopterus pergamentaceus Cuvier	10	403*A*	403*A*	4	1	
Cirratulus grandis Verrill	4	475*A*	475*A*	8	3	
e *Clymanella torquata* (Leidy)	13	487*A*	487*A*	38	.3	
Cystenides gouldii Verrill	3		35	256*A*	4	
Diopatra cuprea (Bosc)	17	280*A*	478*A*	30	.2	
Glycera dibranchiata Ehlers	9	498*A*	498*A*	498*A*	.7	.4
Harmothoe imbricata (L.)	7	228*A*	228*A*	4	.5	
Hydroides hexagonus Bosc	12	260*A*	260*A*	105	.5	
Laonice viridis (Verrill)	8	230*A*	230*A*	230*A*	230*A*	
Lepidonotus squamatus (L.)	13	474*A*	474*A*	379*A*	.3	
Limulus polyphemus (L.)	13	191*A*	191*A*	192*A*	192*A*	40
Lumbricillus agilis Moore	5	8	100	130	.3	
Maldane urceolata (Leidy)	1			8		
Nephthys bucera Ehlers	2	356	115			
Nereis virens Sars	13	236+	259*A*	500*A*	500*A*	3
Phascoloscoma gouldii (Pourtales)	4	252*A*	298	.3	.2	
Pista palmata (Verrill)	5	303*A*		35	26	

SUMMARY.

The ability of *Limulus*, *Phascolosoma*, and eighteen marine annelids to survive in various dilutions of sea water was studied. Most of the animals lived for a week or two in a mixture of three fourths sea water and one fourth fresh; several species lived in one half sea water. *Limulus*, *Laonice*, and *Nereis* lived and were active for periods of two to three weeks in one fourth sea water, but died in weaker solutions.

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THE EFFECT OF THE QUANTITY OF CULTURE MEDIUM ON THE DIVISION RATE OF *OXYTRICHA*.

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The hypothesis advanced by Robertson (1923) that unicellular organisms produce an autocatalytic substance which aids in bringing about their own division, has been subjected to investigation by a number of workers who find little if any evidence supporting Robertson's view. In a recent paper Greenleaf (1926) presents the results of a series of experiments concerned with the effect of the amount of culture medium and cell proximity on the division rates of various Protozoa. From these results he is unable to find any evidence in favor of the autocatalytic effect. On the other hand he finds evidence corroborating the findings of Woodruff (1911), namely, that in small quantities of culture medium the division rate of isolated individuals is retarded. This is due, according to the author, to the accumulation of excretory products and their greater concentration in small amounts of medium. This retardation effect is lessened as the quantity of medium is increased.

These differences in findings leave the problem still open for investigation. It is the purpose of this brief article to set forth the results of some experiments made in an attempt to determine the relation of the amount of culture medium to the division rate of certain protozoa. No attempt has been made at this time to go into all the phases of the whole subject. The experiments were carried on with a species of *Oxytricha*. One of these organisms was isolated from a laboratory aquarium into ten drops of sterile .05 of one per cent. beef extract solution, on a depression slide. This was kept in a moist chamber made of a covered glass disk 7 x 4 x 4 inches. A pure line was established by reisolating every day for several days. When it was desired to start the experiments one or more slides were allowed to stand

48 hours or until there were sufficient organisms in one slide to start the desired number of cultures. All cultures of a given experiment were therefore descendants of a single individual isolated 48–72 hours before.

Oxytricha was chosen after a number of preliminary trials with various ciliates, because of its rapid division rate. At laboratory temperature there are usually two or more divisions in 24 hours and in rare cases as many as six generations have been produced in a day. The large number of individuals produced seemed to make the organism a more favorable subject for these experiments than other more slowly dividing ciliates.

The experiments consisted in comparing the division rate of sister cells in four and ten drops of .05 of one per cent. of sterile beef extract.

In order to keep the organisms in as near the same atmospheric environment as possible slides with two depressions were used. One depression contained the organism in four drops, the other the culture of ten drops. Four slides were used in the earlier experiments, but the number was later increased to ten. The pipettes for measuring the quantity of culture medium were drawn out so that one drop was about one one-hundredth cubic centimeter.

The first series of experiments was carried on at room temperature but as this fluctuated greatly it was decided that controlled temperature conditions were essential for reliable result. For temperatures above ordinary room temperature a Freas electric incubator was used and for temperatures below room temperatures a Cenco water bath was used, in which the moist chambers were sunk almost to their tops in the water and a constant temperature of 18° C. was maintained.

In all cases counts were made at the end of the 24-hour period. One or more slides were set aside to develop "seed" for the next day's experiments. Division rate was measured by the total number of organisms produced in 24 hours from an isolated individual in a given amount of culture medium.

In making the isolations care was taken to take individuals of as near the same size as was possible. In a culture divisions do not occur simultaneously and there are therefore small individuals

—the result of very recent division, large individuals almost ready to divide, and a majority of an intermediate size, probably those full grown. This latter group was selected for the experiments. All organisms used in one experiment were of the same line of protoplasm and as near the same age with respect to the last division as could be determined.

SERIES I.

Series I. was carried on at laboratory temperature which fluctuated very much from day to night, varying probably from 16° C. to 22° C. The results, however, are fairly constant.

Experiment.	4 Drops.		Experiment.	10 Drops.	
	No. of Cases.	Total No. Individuals.		No. of Cases.	Total No. Individuals.
1	4	28	1	4	27
2	4	10	2	4	8
3	4	13	3	4	13
4	4	22	4	4	15
5	4	24	5	4	14
6	4	20	6	4	16
7	4	27	7	4	36
8	4	45	8	4	20
9	4	15	9	4	14
10	4	15	10	4	16
	40	219		40	179
	Average, 5.48			Average, 4.47	

This indicates that at room temperature the individuals in four drops have a much higher division rate. The average division rate is 24.6 per cent. higher in the former than in the latter.

SERIES II.

Series II. was similar to Series I., except for the fact that the temperature was maintained at about 24° C. The division rate was therefore higher than in Series I.

Here again the four drops show a higher division rate than ten drops. The average for four drops is 12.3 per cent. higher than for ten drops.

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4 Drops.			10 Drops.		
Experiment.	No. of Cases.	Total No. Individuals.	Experiment.	No. of Cases.	Total No. Individuals.
11	4	50	11	4	28
12	4	21	12	4	17
13	4	26	13	4	27
14	4	37	14	4	35
15	4	38	15	4	48
16	4	31	16	4	28
17	4	10	17	4	15
18	4	76	18	4	47
19	2	59	19	4	82
	34	348		36	327
	Average, 10.2			Average, 9.08	

SERIES III.

Since the difference in division rate in the two given amounts was larger in the lower (room) temperature (Series I.) it seemed advisable to compare the differences in rates at different controlled temperatures. Therefore, a series of experiments was run with individuals grown in four and in ten drops at 18° C. and at 23° C. The organisms used in any one day were from the same isolated individual. They were presumably as near the same age as it was possible to get them and all had been under identical environmental conditions.

18° C.						23° C.					
4 Drops.			10 Drops.			4 Drops.			10 Drops.		
Exp.	No. Cases.	In-divid.	Exp.	No. Cases.	In-divid.	Exp.	No. Cases.	In-divid.	Exp.	No. Cases.	In-divid.
B	10	19	B	9	15	A	7	41	A	10	45
D	9	38	D	10	32	C	10	59	C	10	77
F	10	32	F	10	31	E	10	83	E	10	80
H	10	46	H	10	36	G	10	103	G	10	82
L	10	44	L	10	34	K	10	138	K	10	142
N	10	26	N	10	24	M	10	87	N	9	60
P	9	36	P	10	29	O	10	97	O	10	76
	68	241		69	201		67	608		69	562
	Average, 3.54			Average, 2.91			Average, 9.07			Average, 8.1	

As in Series I. and II. the division rate in Series III. is higher in four drops than in ten drops. At the lower temperature the difference in the division rate in the two groups is greater than it is at the higher temperature. In the former the organisms in four drops have a higher rate by 21.64 per cent. while in the latter the four drops have a higher rate by 10.19 per cent.

In all of these experiments the division rate in four drops exceeds that in ten drops by more than ten per cent. If we consider only the amounts of culture medium and disregard temperature the results are as follows:

4 Drops.			10 Drops.		
Series.	No. of Cases.	Individ.	Series.	No. of Cases.	Individ.
I........	40	219	I........	40	179
II........	34	348	II........	36	327
III 18°....	68	241	III 18°....	69	201
23°....	67	608	23°....	69	562
	209	1,416		214	1,269
	Average, 6.77			Average, 5.93	

The division rate in four drops is considerably higher than that in ten drops—a difference of 14.17 per cent. This result is perhaps more significant than the others since there are over 200 cases considered.

The results of these experiments have a direct bearing on two important problems. The first of these is the question of the autocatalase. Robertson (1923) quite definitely showed that with the infusorian *Enchelys*, isolated in a limited amount of culture medium, there was an increasing reproductive rate during the second twenty-four hours of a culture over that of the first twenty-four hours. He also found that, within certain limits, division rate of *Enchelys* was higher in the first twenty-four hours in small quantities of culture medium than in larger quantities and that individuals isolated into one cubic centimeter or more of medium rarely survive.

In his interpretation of these and other experiments Robertson was forced to the conclusion that "the only possible inference that can be drawn from this phenomenon is that infusoria discharge into the culture medium some substance which accelerates their own multiplication."

As further test of this theory he showed that reproduction would occur in sterile culture medium or in distilled water and cites the experiments of Peters (1921) with *Colpidium* to show that bacteria as food are not essential for reproduction of infusoria if the proper ingredients necessary for growth are put into the medium.

The second question for consideration is that concerning the effect of the excretory products on the division rate of protozoa. Woodruff (1911) has shown that the rate of division is very markedly lowered by the accumulation of excretory products. Greenleaf (1926) has taken up the problem and using several different infusorians arrived at the same conclusions as those reached by Woodruff fifteen years before. These results are in direct contradiction to those of Robertson and Greenleaf concludes that as far as the organisms with which he worked are concerned there is no evidence of the autocatalytic effect. He further concludes that infusoria tend to multiply faster in larger amounts of culture medium and that one individual in a given amount of medium multiplies faster than each does when two individuals are in the same amount. This difference is explained on the basis that two organisms would raise the concentration of the excretory products to a degree injurious to themselves more rapidly than a single individual would do.

The results of the experiments upon which this paper is based seem to favor the idea advanced by Robertson. In all series in which four and ten drops of culture medium were used the average division rate in four drops was higher than in ten drops. Since the culture medium was sterile when the organisms were introduced, the question of food does not seem to be one of prime importance in our consideration. The most plausible explanation therefore is that *Oxytricha* produces a substance which is liberated into the surrounding medium and which reacts on the infusorians to bring about cell division, and that the concentration of the substance necessary for bringing about cell division is reached in the smaller amount of culture medium before it is in the larger amounts.

The conflicting conclusions reached by various workers leave the question still unsolved. It is possible that the various results have been due to the fact that organisms with a high division rate show the effect of the autocatylitic substance more readily than do organisms which divide only once or possibly twice in twenty-four hours. Robertson used *Enchelys* which divides many times a day. Woodruff and Greenleaf used larger infusorians which have a much slower rate of division. Between these extremes *Oxytricha* has an intermediate division rate and in the experiments cited undoubtedly shows a higher average rate of division in the smaller amounts of culture medium. There seems to be no explanation of this so satisfactory as that suggested by Robertson for *Enchelys*, namely, that *Oxytricha* produces a substance which reacts on the organism to bring about division, and that the concentration of this substance is reached in sufficient quantities to bring about cell division in small quantities of culture medium before it is in greater quantities.

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CONJUGATION, DIVISION, AND ENCYSTMENT IN *PLEUROTRICHA LANCEOLATA*.[1]

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Introduction.

Evidence has been steadily accumulating of recent years that many closely related species differ from each other in number of chromosomes, the numbers often being multiples, or multiples of some common factor. Because of this fact and because the processes of conjugation have been thoroughly studied in only a very few hypotrichs, the present investigation was undertaken. *Oxytricha fallax* and *Pleurotricha lanceolata*, although placed in different genera, are morphologically quite similar, and it was thought that a comparison of the two species with respect to chromosome number and details of conjugation would be of interest. It has been shown before in at least one case (*Chilodon uncinatus*, MacDougall, 1925) that new species may arise de novo from old ones among the protozoa, the chief differences being in chromosome number, and it is not unlikely therefore that this often happens in nature. A study of division and encystment was undertaken as a natural corollary.

Pleurotricha lanceolata and Similar Species.

Pleurotricha lanceolata was first described accurately by Stein in 1859, although two other species which he regards as the same had been described previously—*Stylonychia lanceolata* and *Keratium calvitium*, the former by Ehrenberg in 1832 and the latter by Müller. But Ehrenberg's organism had 16 to 18 cirri on the dorsal surface as well as the full complement on the ventral surface, and Müller failed to see the abdominal cirri, so that priority of description and name belongs to Stein.

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Pleurotricha lanceolata was also described and figured by Kent (1880 to 1882), but his description is slightly different from that of Stein. In addition to the double row of marginal cirri on the left-hand side, Stein shows a very short third row in the middle region of the body which Kent omits, as well as the anterior third of the inner marginal row.

The race which I have used (Fig. 1) fits Kent's description very closely, but is only about half as large, and the inner marginal row of cirri is found only in the middle third of the body. Stein gives the size as varying between 83 and 143 μ, and Kent makes it just about twice as great. I have found the average size to be about 140 μ, the extremes being 100 to 165 μ, if exconjugants which are always exceptionally small are omitted.

Oxytricha bifaria (Stokes, 1887) also resembles *Pleurotricha lanceolata*, differing principally in having only a single marginal row of cirri extending around the body, and in having eight instead of six cirri on the anterior quarter.

There is also a considerable resemblance between *Oxytricha fallax* and the chief species under discussion. The former has five anal cirri arranged in an oblique row, the two posterior extending only slightly beyond the body margin.

Material and Methods.

The cultures were started from a single individual obtained from a mass culture of leaves and swamp water, which in turn was secured from Orient Springs, near Amherst, Massachusetts, November 5, 1925. These cultures were continued until May 1927, the medium being hay infusion, to which in some cases a small amount of peptone was added.

All preparations were fixed with Calkins' modification of Schaudinn's fluid, and stained with hemotoxylin, both the long and short methods being used.

Division.

The first indication of division is seen in the macronuclei, in each of which a nuclear cleft, or kernspalt, appears at the extreme outer ends, and this gradually moves across the nuclei to finally disappear at the inner ends. Simultaneously, or very shortly afterward, a new adoral zone begins to form in the midregion of the body. This stage of division is shown in Fig. 2.

Very soon the micronuclei begin to enlarge and a clear space, or halo, appears about each. At about the same time a division center appears and divides, the two products remaining connected by a centrodesmose (Figs. 3 and 4). I have never been able to detect this endosome in the resting micronucleus however, and it is difficult to observe even in division. As the dividing nucleus enlarges it elongates, and the chromatin appears to be arranged in very fine strands, some of which seem to be in reality rows of granules (Fig. 4 *A*). At this time it stains very faintly. The chromatin next appears to become more or less concentrated at the poles (Fig. 4 *B*). As all this goes on the macronuclei change their shape, becoming almost round and the kernspalt finally passes off the inner ends (Figs. 4, 5, 6). The new adoral zone meanwhile increases in size, and new cirri may be seen developing beside the old ones of the old adoral zone (Fig. 6). In Fig. 5 the new ventral cirri may also be seen close to the adoral zones, where they first arise. As the time approaches for final separation of the daughter individuals these cirri gradually move to more nearly their normal positions (Fig. 11).

In the meantime the chromatin of the micronuclei appears to condense into discrete masses, which take the form of threads of varying length (Fig. 5 *B*). These gradually combine, until the nucleus appears to be composed of closely packed and more or less tangled threads, as in Figs. 5 *A* and 6 *B*, and finally a definite spindle appears (Fig. 6 *A*). The poles of the latter move apart and a very characteristic figure is produced which is shown in Fig. 7 *C*. Here the chromosomes appear as heavily staining Vs, the limbs of which are connected by heavy and deeply staining strands. Apparently division is really longitudinal, the two daughter chromosomes taking on a V shape and appearing connected at the ends for a while thereafter. Their final separation is shown in Fig. 7 *A*. Fig. 8 *A* shows a somewhat later stage of the metaphase.

At about this time the macronuclei begin to elongate (Fig. 8) and the remaining stages in division are passed through very rapidly, the time required being about fifteen minutes at ordinary temperatures, as compared to more than an hour for the preceding steps. The micronuclei enter the anaphase, which is quickly completed, requiring only a very few minutes, and which may be seen in Figs. 9, 9 *A*, 9 *B*, 9 *C*. The telophase is shown in Figs. 10, 11, 11 *A*, 11 *B*, and 12. The macronuclei divide at this time (Figs. 11 and 12), and the parent animal very shortly separates into two daughter individuals (Fig. 13).

Discussion.

The process of division in ciliates is so well-known that few comments are necessary, since different species differ only in minor details, and fission in *Pleurotricha lanceolata* follows the general plan. There are, however, a few points in which this species differs from others, and in other respects there are interesting resemblances.

The presence of a kernspalt is said to be common to all hypotrichous ciliates, even during the major part of the resting stage (Calkins, 1919), but I have never been able to see it in *Pleurotricha* except when the animal was about to divide, as shown by the presence of a rudimentary adoral zone. This happens even before there is any visible change in the micronuclei.

Apparently the early appearance of a new cytostome with its accompanying peristomial apparatus is characteristic of most dividing infusoria, *e.g. Uroleptus mobilis* (Calkins, 1919), *Chilodon uncinatus* (MacDougall, 1925), although there are other cases in which its development is more delayed as in *Paramœcium trichium* (Wenrich, 1926).

It seems to be generally accepted that in division there is a complete reorganization of the entire cell, extending even to the disappearance and subsequent reformation of all the organelles, including trichites in organisms which have them, undulating membranes, cirri, and even cilia. According to Wallengren (1900) the cirri of the old adoral zone in hypotrichous ciliates are gradually absorbed as new ones take their places, and I have been able to confirm this observation for *Pleurotricha*. That this reorganization does not always extend to the old oral apparatus, however, is indicated by Wenrich (1926) who was unable to find evidence that the old cytostome disappeared and was reformed in *Paramœcium trichium*.

It is a remarkable fact that all the cirri, with the exception of the marginal ones, arise in the immediate neighborhood of the adoral zones, and indeed the first indications of their appearance are visible very shortly after the adoral zone itself appears. That this was the case in the Oxytrichidæ was noticed by Sterki (1878), and has been noted for other groups by other investigators. MacDougall (1925) found that new cilia in a dividing *Chilodon uncinatus* first appear near the new pharyngeal baskets.

The presence of a centrosome which divides as an initial step in division has until recently not been noted in ciliates. It was reported by Calkins in the first maturation division of *Uroleptus mobilis* however (Calkins, 1919), although he evidently did not see it in ordinary division, and MacDougall found it in *Chilodon uncinatus*. To this list can now be added *Paramœcium trichium* (Wenrich, 1926), and *Pleurotricha lanceolata*.

Encystment.

Encystment is a common occurrence in the life cycle of *Pleurotricha bifaria*, although what the conditions are which determine its occurrence I have been unable to discover. It may occur in old cultures, and is perhaps most frequent under those circumstances, but it also occurs in pedigreed drop cultures, which are changed every day, and in which the division rate is rapid. Of four individuals which have arisen by division from a single ancestor during the preceding twenty-four hours it is not uncommon to find one or two encysted, while the others continue to divide actively.

The account of nuclear phenomena which follows is admittedly inadequate, but it is given since no other description has been made of this stage in the life cycle of this particular species, and because the history of the cytological changes which occur in ciliates during encystment is notoriously incomplete.

The first change appearing in an individual about to encyst is like that occurring in other infusoria under similar circumstances. The shape becomes spherical, the contractile vacuole gradually ceases to pulsate, the food vacuoles and all the organelles disappear (Fig. 14). The next step is the secretion of the cyst wall, shown in Fig. 15. It is thick, quite pliable but very tough, and without visible openings. The outside is covered with irregular, short spines. Such a cyst is shown by Stein (1859) for *Pleurotricha lanceolata*, but Cienkowsky (1855), who gives three figures of the cyst formation of *Stylonychia lanceolata*, does not show the spines. Nevertheless these two species are regarded by Stein as the same, as already stated.

Soon (probably under normal conditions in about twenty-four hours) the old macronuclei, and apparently one of the micronuclei, are bodily extruded through the cyst wall, although the opening through which they pass is never visible under any other circumstances (Fig. 16). This leaves the encysted animal in the condition shown in Fig. 17. The remaining nucleus then proceeds to divide but I am unable to say much as to the details of this division. Apparently some of the micronuclei shown in Figs. 18 and 19 are in process of division, and the process appears to be a typical mitosis. As to how a cyst in the condition of that in Fig. 19, just referred to, reaches that shown in Fig. 20 I am unable to say. Cysts are exceedingly difficult to stain and destain at this stage.

Figs. 20 and 21 show the final stages in the reorganization. As may be seen there has been a great decrease in the quantity of cytoplasm, and a corresponding decrease in chromatin. Animals at this stage may be seen swimming about within the cyst, but I have never been able to get them to leave the cyst, despite washing, prolonged observation in cultures in which the media was changed daily, and attempts to rupture the cyst wall artificially. The latter always failed because of the tough pliable nature of the wall. Both distilled water, tap water and Ringer's solution were used for washing.

Moore (1924) found that *Spathidium spathula* behaved in a similar fashion, though she was more successful in liberating encysted individuals by puncturing the cyst wall. Under ordinary circumstances few encysted animals would excyst, and drying, which Calkins (1919) found necessary to induce the excystment of *Uroleptus*, and the addition of fresh medium, found efficacious by Moody (1912) in securing the excystation of *Spathidium*, both proved unsuccessful.

But the cytological changes which occur during the encystment of *Spathidium spathula* appear to be markedly different from those which take place in *Pleurotricha*. There is no extrusion of nuclear or cytoplasmic material from the cyst in Spathidium, but the macronuclei degenerate and finally disappear, the fragments being absorbed by the cytoplasm. The micronuclei remain practically unchanged. Shortly before redifferentiation occurs, preparatory to leaving the cyst, new macronuclear anlagen appear which strongly resemble those formed in the late stages of conjugation. It is possible that more study would show that the new macronuclei arise in a similar way in *Pleurotricha*, but I have seen no indication of it in any of my preparations.

That an extensive nuclear reorganization takes place in many infusoria during encystment has been known for a long time, but I have found no mention of the extrusion of nuclear material in any other species, although Prowazek (1899) believed that such a phenomenon occurred during conjugation. Fermor (1913) found that in *Stylonychia* there was a fusion of the two micronuclei previous to the formation of a new nuclear apparatus, and it is possible that the same thing occurs in encysted individuals of *Pleurotricha*. But in one instance I have actually observed one of the two micronuclei being extruded along with the macronuclei.

CONJUGATION.

During the eighteen months in which the cultures were maintained conjugation occurred but rarely, and epidemics of it were never observed. Even in mass cultures containing thousands of individuals it was usually necessary to look for an hour or more to obtain a few pairs. Because of the small number of conjugants no sections were made and all the results herein described were obtained from whole mounts, fixed with Calkins' modification of Schaudinn's fluid, stained with hematoxylin, and mounted individually. Various methods of inducing conjugation have been suggested by investigators who have found them more or less useful with certain species, and a number of these methods were tried in this case, but with practically no success.

Conjugating individuals fuse by the adoral surfaces (Fig. 22), the entire peristome of one member of the pair disappearing completely. There seems to be no reason why it cannot function in the other conjugant but there is very little evidence that it does so, for food vacuoles become fewer in number as conjugation progresses, and exconjugants are always small. The time from fusion to final separation varies from eighteen hours to five days, but the usual duration is twenty-four hours.

Behavior of the Micronuclei.

The micronuclei normally go through three maturation divisions, only two taking part in each. The others degenerate more or less rapidly, although some may persist even after the interchange. The pronucleus usually undergoes two cleavage divisions. Of the four products one enlarges and eventually gives rise to the macronuclei of the reorganized exconjugant, one degenerates, and the remaining two form micronuclei.

The First Maturation Division.

This division requires more time than any of the others—at least eight hours—and is also strikingly different in type. The micronuclei at first show no change but soon increase in size, become surrounded by a clear space, or "halo," and stain more faintly than usual. The chromatin takes on a finely-granular appearance. Shortly an endosome, or division center, appears, and in favorable preparations two may be seen (Fig. 28 *A*, *C*), but I have never been able to detect an intradesmose connecting them, although in vegetative division and in the other divisions of conjugation it can often be seen. This division center increases in size until it becomes hemispherical and stains very heavily (Fig. 28 *C*, *D*). In the meantime spindle fibers begin to appear, and the spindle takes on the typical parachute appearance (Fig. 28 *C*, *D*, *E*). The chromatin in the expanded top of the parachute, which is at first composed of very small, dimly staining granules, condenses into larger granules which stain more heavily and these pair to form heavily staining chromosomes of dumbbell shape. These granules are altogether too numerous to count, and in most preparations this is also true of the chromosomes, but I have made counts in a few cases which will be discussed at greater length below. The chromosomes now move to the center of the spindle and divide longitudinally (Fig. 28 *F*, *G*). Stages of the anaphase and telophase are shown in Figs. 28 *H* to *M*. The most characteristic feature of this division, aside from the dumbbell shape of the chromosomes, is the way in which they lag and the peculiar curve at the poles of the spindle.

The Second Maturation Division.

This follows rapidly on the first, and is over in much less time, with the incidental result that material showing it is difficult to obtain. It is also of very different character. The micronuclei which are to divide enter the prophase almost before the telophase of the preceding division is complete (Fig. 32 *A*). At this stage they stain faintly, and rows of granules appear which are arranged in a more or less "whorled" fashion (Fig. 32 *B*). Fig. 32 *C* represents a stage which is seldom seen, but it is presumably earlier than those shown in the two preceding figures. The nucleus next enlarges and the rows of granules become threads (Fig. 32 *D*). This may well be in reality a leptotene stage, since reduction occurs during this division. There is soon evidence of a definite spindle which is at first of a peculiar oval shape (Fig. 32 *E*, *F*). I have been unable to get preparations showing the metaphase and later stages of the anaphase, but the early anaphase is shown in Fig. 32 *F*. I have made a number of counts of the number of chromosomes concerned in this division, the average result being forty. Two stages of the telophase are shown in Fig. 32 *G* and *H*.

The Third Maturation Division.

The third division differs in type from both the preceding, and requires more time than the one just described. The micronuclei which are to divide enlarge, and become very finely granular. I am uncertain as to whether there is an intradesmose connecting the products of the division of the intranuclear endosome, but I believe that there is. What is apparently one end of it can be seen in Fig. 36 *A*. These granules now condense to form rows, and the latter in turn become much coiled chromosomes which often appear double (Fig. 36 *B*, *C*). The metaphase is shown in Fig. 36 *D*, and is considerably like that of ordinary vegetative division. The chromosomes in both the second and third maturation divisions are rod-shaped, in contrast to the dumbbell shape which they have in the first, and the V and irregular rod shape of the cleavage divisions. The long-pointed anaphases are characteristic of the third division, and long drawn-out telophase (Figs. 36 *E* to *I*).

The interchange is a rapid process, and the wandering nuclei do not appear to differ in any respect from the stationary ones. New adoral zones appear at this time or very early during the first cleavage division (Fig. 37).

The First Cleavage Division.

The amphinucleus is shown in Fig. 40, and is apparently divided into two parts, doubtless representing the two pronuclei. The stages in the first cleavage division are shown in Figs. 41 *A* to *H*. The nucleus, which increases very much in size shortly after fusion, becomes smaller, and forms a peculiar sort of spindle (Fig. 41 *A*), in which the chromatin appears to be condensed into a heavy ribbon, twisted more or less upon itself, and quite definitely double. Chromosomes soon appear which are arranged in bouquet fashion, about a small endosome, and which at this stage seem to be, in some cases at least, in pairs (Fig. 41 *B*). They then straighten out and form a characteristic spindle which has at first two definite parts, possibly representing the two pronuclei (Fig. 41 *C*). At this time the chromosomes are definitely double, and apparently twisted about each other. The metaphase is figured in 41 *D*, and the anaphase soon follows. Apparently the chromosomes in the later stages of this division are shaped like very acute Vs. Figs. 41 *E* to *H* show the anaphase and telophase. The former is rather characteristic in its early stage because the chromosomes are so widely separated within the receding plates.

The Second Cleavage Division.

The first cleavage division which has just been described is a rapid process, but the second one is much slower. A division center appears, as in two of the three maturation divisions, and divides, the products remaining connected by an intradesmose (Fig. 46 *A*). The nucleus then becomes elliptical, and the chromatin becomes arranged in long deeply-staining strands which are at first quite regular in arrangement (Fig. 46 *B* and *C*). These elongate and become twisted (Fig. 46 *D*, *E*), some of them appearing double. The whole spindle resembles nothing so much as a tangled skein of yarn at this stage, and possibly the stage represented in Fig. 46 *D* is in reality a spireme. The strands in the following figure are definitely polarized and probably are really chromosomes. The metaphase is shown in Fig. 46 *H*. It does not differ very much from that in the first cleavage division. The anaphase is very peculiar, as can be seen from Figs. 46 *J* to *M*. At the poles of the spindle a "cap" of very deeply-staining chromatin is formed and the chromosomes lose the definite shape which they had in the metaphase, apparently coalescing into more or less irregular masses which take a very heavy stain. These are very characteristic and make the later stages of the second cleavage division differ from all the others. The strands persist for some time in the anaphase (Fig. 46 *L*, *M*).

The Occasional Third Cleavage Division.

Following the second division there may in some cases be another, but when it occurs only two of the four nuclei take part as a rule, so that exconjugants in which this third division has taken place have six nuclei, rarely eight. All the stages of this division which I have observed are like corresponding ones of the second division.

The Behavior of the Macronuclei.

The anterior of the two macronuclei elongates during the maturation divisions, and may occasionally divide by a process of mass division, but only in very rare cases. At the same time it undergoes a slow fragmentation, so that the cytoplasm usually has a number of heavily staining masses of chromatin at this time, which often simulate degenerating micronuclei derived from previous divisions. The posterior macronucleus may elongate slightly, but I have never observed it to divide and it undergoes much less fragmentation than the other.

The chromatin fragments and degenerating micronuclei usually disappear about the time of the interchange, or soon afterward, but may in some cases persist until after the cleavage divisions.

Reorganization of the Exconjugant.

Reorganization begins before separation, and usually requires several days. One of the four nuclei resulting from the second cleavage division becomes very large and coarsely granular, and at the same time loses much of its capacity to stain with hemotoxylin. It is destined to form the macronuclei of the reorganized exconjugant and is shown in Figs. 47, 48, 49, 50, 52 and 53. This nucleus divides at the first division of the exconjugant, and then divides again without corresponding division of its possessor, thus restoring the normal macronuclear condition (Fig. 55).

But it is apparently possible for reorganization to become complete without prior division of the exconjugant, and an individual in which this has happened is shown in Fig. 54. It may be that this can happen in individuals in which there has been a third cleavage division.

The micronuclei of the reorganized conjugant are derived directly from two of the three which remain from the second cleavage division. The third persists for a time but eventually degenerates. As to what happens when there have been three cleavage divisions I am unable to say, since I have been unable to observe enough cases.

The remains of the old macronuclei persist for a day or two as more or less circular, deeply staining and vesicular masses of chromatin (Figs. 49 to 52), but they eventually disappear completely, leaving no trace (Fig. 53).

The Number of Chromosomes.

In order to determine the number of chromosomes a large number of counts were made in several different stages, particularly from an especially favorable preparation showing the early anaphase of the first maturation division, and from several preparations of the anaphase of the third maturation division.

Since the total number of chromosomes is so large in the anaphase of the first maturation division accurate counts are difficult, but ten were made as a check for the other counts described below. The mean of these ten counts was eighty-six, which would indicate that the diploid number is forty-three. The standard deviation was found to be 15.4, the coefficient of variation 17.9, and the probable error ± 3.46. There is no doubt therefore that the counts are very significant.

A few counts were made of the chromosomes in the early anaphase of the second maturation division, and the average was close to forty, again indicating therefore that this figure is close to the diploid number.

Since the most favorable preparations for chromosome counting were those of the third maturation division these were given the most study. To insure results as free from error as possible seventy counts were made from several preparations showing the anaphase of this division. A curve was constructed of these counts and the mode found to be 19. The mean was 19.62, and the probable error ± .295. The coefficient of variation was 18.6 per cent., and the standard deviation 3.66. It is therefore certain that the counts are highly significant, and there is no doubt that the haploid number of chromosomes is close to twenty, with a high degree of probability that it is exactly that number, making the diploid number forty.

Since the number of chromosomes in *Oxytricha fallax* is twenty-four (Gregory, 1923), there is therefore no obvious relation as far as number of chromosomes is concerned between this species and *Pleurotricha lanceolata*.

Discussion.

Although only a relatively small number of ciliates have been studied with reference to the phenomena of conjugation the process appears to be essentially the same in all. From his own observations Maupas (1888) divided it into eight phases—*A*, the period of preparation preceding the first meiotic division; *B*, the first division; *C*, the second division; *D*, the third division; *E*, the interchange and fusion of the pronuclei; *F*, the first cleavage division; *G*, the second cleavage division; and *H*, the period of reorganization preceding the first fission of the ex-conjugant.

These phases have been shown to hold for all infusoria so far studied, including *Pleurotricha lanceolata* which is the subject of this paper, although in a few forms such as the Vorticellidæ, Ophryoscolecidæ, and *Euplotes patella*, there are one or more preliminary divisions before the meiotic divisions begin. (In the case of the Vorticellidæ this happens only in the case of the microgamete).

Ciliates as far as at present known fall into two classes according to the behavior of the micronuclei in the first phase—those which undergo a prophase like *Paramœcium*, the micronucleus being drawn out into a crescent, and those in which a parachute or candelabra-like figure is formed, to use Calkins' term. To the last group belong *Onychodromus grandis* (Maupas, 1888), *Bursaria truncatella* (Prowazek, 1899), *Didinium nasutum* (Prandtl, 1906), *Anoplophrya branchiarum* (Collin, 1909), *Uroleptus mobilis* (Calkins, 1919), *Oxytricha fallax* (Gregory, 1923) and *Chilodon uncinatus* (MacDougall, 1925). The character of the first division, already described, makes it necessary to add *Pleurotricha lanceolata* to this list.

As might perhaps be expected from the close morphological resemblance of *Oxytricha fallax* and *Pleurotricha lanceolata* the details of the conjugation process are much alike, and while they are also similar to those which occur in *Uroleptus mobilis* as described by Calkins, the resemblance is much less striking.

The formation of the parachute preliminary to the first maturation division more nearly resembles the corresponding stages in *Uroleptus* than in *Oxytricha*, but differs from both. In the latter, although the parachute fibers are focused on a single granule derived by division from the endosome just as they are in *Pleurotricha*, there is also a centrodesmose connecting the two halves of the endosome which I have never been able to observe in *Pleurotricha*. In *Oxytricha* there appears to be no endosome, and the place of the basal granule is taken by a row of granules.

The chromosomes in this division appear to be formed by the fusion of the granules into which the chromatin is divided early in the prophase, but whether the number of these granules bears any constant relation to the number of chromosomes, as Gregory believes it does in the case of *Oxytricha*, I am unable to determine. These chromosomes which at first make up the top of the parachute appear to move down the fibers until they reach the center of the spindle when they form an equatorial plate. The other pole of the spindle appears to be formed by one of the two halves of the endosome which remains in the top of the parachute, the other as already stated, forming the granule at its base. This is apparently the same as in *Uroleptus*.

The number of nuclei taking part in any of the three maturation divisions is apparently never greater than two in the case of *Pleurotricha*, although forms in which there are three micronuclei occur. Of the four products of the first division all but two degenerate, and the same thing happens after each of the other two maturation divisions. The number of nuclei taking part in each maturation division in *Oxytricha* appears to be variable, although there are always at least two. In other multinucleate ciliates which have been studied there is considerable variation in this respect. In *Bursaria* all the sixteen or more nuclei may take part in the first meiotic division (Prowazek, 1899), and in *Uroleptus* there may be two, three or four primary spindles (Calkins, 1919), but these are exceptions.

The second maturation division is apparently of different character from the first in most ciliates, and lacks the elaborate preliminaries of the latter, with the result that it requires much less time. The micronuclei in most cases do not return to the resting stage between the first and second division, although there are exceptions (*e.g.*, *Chilodon*, MacDougall, 1925). In all these respects therefore *Pleurotricha lanceolata* is typical.

In nearly all cases in which a careful study has been made reduction has been found to take place in the second meiotic division, although the first division was thought to be the reducing division in *Paramœcium caudatum* (Calkins and Cull, 1907), and in *Oxytricha fallax* (Gregory, 1923). But Dehorne (1920) regards the third division as the one in which reduction occurs in the former form. In the case of *Pleurotricha* there is no doubt but that it occurs in the second, for the following reasons: The total number of chromosomes taking part in the first division in the beginning anaphase has been determined to be in the neighborhood of eighty, as already stated, whereas there are only half as many concerned in the second division, and the average number moving toward each pole of the third maturation spindle has been determined, as stated above, to be about twenty.

The only protozoa in which reduction is known to occur at any other time (with the two exceptions already mentioned), are *Diplocystis* (Jamieson, 1920), and *Aggregata eberthi* (Dobell, 1915). It is suggested by Dobell and Jamieson that this may be a universal occurrence among the Telosporidia.

The third maturation division and the interchange take place exactly as in *Oxytricha*, and are very much alike, even to details in the form of the spindle. Nothing is said as to the existence of a division center or intradesmose however. The dumbbell character of the chromosomes is not lost until the third division in the case of *Oxytricha*, and it is stated that the chromatin streams toward the poles in the form of granules. I find that the chromosomes are definitely rod-shaped in *Pleurotricha*, in both the second and third divisions, although the rods are heavier and shorter in the former, and may give place to rows of granules in the late anaphase stages of the latter. The dumbbell character is never regained after the first of the three maturation divisions.

The two cleavage divisions follow the usual course, again resembling closely those in *Oxytricha*, although not many details are given in Gregory's paper. The products of the second cleavage division are equal as in *Oxytricha*, but differ in this respect from the state of things in *Uroleptus mobilis*, in which one of the two nuclei derived from the first division gives rise to the macronuclei and the other to the micronuclei of the reorganized exconjugant (Calkins, 1919). I have found no other case in which the anaphase of the second cleavage division resembled that in *Pleurotricha* however, and it differs strikingly from that in *Oxytricha*.

The occasional third cleavage division is also a striking characteristic, and as far as I can discover it occurs in no other hypotrich so far described, although it is admitted that the number studied is thus far small. In other forms such as *Paramœcium caudatum* (Calkins and Cull, 1907), and several of the Vorticellidæ, a third division of the amphinucleus occurs, but as a regular thing. In *Bursaria* (Prowazek, 1899), there are four divisions before differentiation occurs.

The behavior of the macronuclei in *Pleurotricha* follows the usual rule—degeneration eventually occurs, although it is not completed until after separation. But division of the macronuclei, although apparently the rule in *Oxytricha*, is very rare in *Pleurotricha*.

The time required for reorganization and the details of the process differ considerably in different cases, but the process as it occurs in *Pleurotricha* is not significantly different from the essential features of reorganization in other species. Prowazek believed that the remnants of the old macronuclei must be extruded bodily from the cell in *Stylonychia pustulata* and *Bursaria truncatella* (1899), but I have seen no evidence of this in the present instance, nor has it ever been observed by anyone else. He based his hypothesis on the fact that nucleins are indigestible.

The Physiological Effect of Conjugation.

The significance of conjugation has been much disputed, one school holding that it is the necessary result of senescence in any given race, and that by it the death of the race could be averted and rejuvenation, expressed chiefly in an acceleration of the division rate, be secured. Maupas held this view and since his time Calkins has been its leading exponent. Another group has regarded conjugation as a process useful chiefly as a means of producing variations, which are much greater and much more frequent in exconjugants than in ordinary vegetatively reproducing individuals. Jennings has been one of the chief expounders of this view.

Many experiments have been tried to settle this question and since the results have differed considerably for different species I have endeavored to find out the effect of conjugation in *Pleuro-*

tricha lanceolata, as measured by the division rate of exconjugants when compared to closely related non-conjugating lines.

The experiment consisted in isolating one hundred exconjugants, and following them daily as long as possible. Of this number 92 per cent. died without division, usually about four days after separation, and of the remaining eight only one gave any evidence whatever of rejuvenation. The others divided two or three times and then all died. This single individual gave rise to a line which divided between two and three times a day for six days, and from then until the death of the line four weeks later, the average daily division rate gradually declined until during the last two weeks it was much less than one per day.

The controls during this period averaged from one to two divisions a day, and showed no evidence of senescence for the six months for which pedigreed cultures were maintained. Both controls and exconjugants were in the same media.

It is realized that one hundred cases is scarcely enough to draw final conclusions from, and yet the percentage of deaths is so high that it does not seem likely that a greater number of cases would have given significantly different results.

Jennings (1913) in a long and comprehensive series of experiments demonstrated that with *Paramœcium* "Conjugation decreases the rate of fission, causes a great increase in variation in fission rate, brings about many abnormalities, and greatly increases the death rate," but Mast (1917), using *Didinium nasutum*, was unable to secure evidence of any such effect. He found that there was no appreciable effect on either death rate, fission rate, or increase in variation of fission rate, thus proving however that there was also no rejuvenation.

Calkins, in a long series of experiments with *Uroleptus mobilis*, has shown that in this species at least conjugation appears to have a genuine rejuvenating effect (Calkins, 1919 and 1926), and Woodruff and Spencer (1924) conclude that conjugation has a similar effect in the case of *Spathidium spathula*, although they prefer the term "high survival value" to that of "rejuvenation."

It has been shown by many investigators working with various species that as culture methods are improved the longevity of cultures without conjugation can often be indefinitely increased, so that it can probably be safely concluded that conjugation is at least not a necessary process if environmental conditions are sufficiently favorable.

Since cultures of *Pleurotricha* have been maintained for eighteen months with very little conjugation (none in the pedigreed lines which were carried for six months), and since in the cases mentioned above none of the exconjugants gave rise to lines which continued for any length of time, although kept under identically the same conditions as controls which maintained a uniform and fairly high division rate, it can be concluded I think that conjugation is not an indispensable part of the life cycle. This conclusion is supported by Baitsell (1914) who carried on experiments of this nature with both *Pleurotricha lanceolata* and *Oxytricha fallax*.

It is nevertheless apparent that a process which is so universal among infusoria as conjugation must serve some useful purpose, and that in nature exconjugants do not always die. Why then do they die in cultures? It is impossible to give a definite answer, but it has been suggested that media which is suitable for active vegetative multiplication may not always be suitable for conjugation and exconjugants, and this may well be the explanation.

Summary.

1. *Pleurotricha lanceolata* is a hypotrichous ciliate belonging to the family Pleurotrichidæ. The species has as its chief characteristic six anal cirri, divided into two groups. The anterior of the two groups consists of four cirri—three very large ones arranged in an oblique row, and a smaller one, a little forward of the posterior end of the row. The second group includes two cirri, both of them very long and projecting well beyond the posterior end of the animal. The usual size of vegetative individuals is 140 μ.

2. The process of division does not differ particularly from that in other infusoria, except that in the very early stages an endosome appears and divides, the products remaining connected by an intradesmose. This has been described in *Paramœcium trichium* but in few if any other species. The process of division is initiated by the appearance of a rudimentary adoral zone and

a kernspalt in each of the macronuclei. The micronuclei divide by typical mesomitosis. When division of the latter is virtually complete the macronuclei follow suit, dividing amitotically, and then the cell itself divides. All organelles appear to be regenerated, the old ones being absorbed.

3. Encystment may occur at any time, and appears to bear no relation to periods of depression, to division or to conjugation. The old macronuclei are extruded bodily from the cell, and a single micronucleus remains. It is uncertain whether the other is always extruded with the macronuclei or fuses with the first micronucleus. An uncertain number of micronuclei are formed from the single remaining micronucleus, and from these the normal nuclear complex is rebuilt, the process being complete at the time the animal is ready to leave the cyst.

4. The nuclear changes which occur during conjugation are essentially the same as those described for other ciliates. There are three maturation divisions, an interchange of pronuclei, and two or rarely three cleavage divisions. The four products of the second division are at first alike, but one soon enlarges and eventually gives rise to the new macronuclei of the reorganized exconjugant. One of the others degenerates and the other two form the new micronuclei. The old macronuclei degenerate after separation of the exconjugants. There may or may not be one division of the latter before reorganization is completed.

5. Reduction occurs in the second maturation division. The diploid number of chromosomes is forty, as nearly as can be determined, and the haploid number twenty. The chromosomes are dumbbell-shaped in the first maturation division, and rod-shaped in the second and third. They are also rod-shaped in the cleavage divisions, but the shape then is unlike that in the maturation divisions or in vegetative division.

6. Each of the divisions in conjugation differs from all the rest and from vegetative division.

7. Conjugation occurs but rarely in the race of *Pleurotricha* used, and under the conditions of culture virtually always results in the death of the conjugants a few days after separation. In only one instance out of a hundred did it result in anything like rejuvenation, and even in this case the daughter race died within a month.

8. Although the details of conjugation are much alike in *Oxytricha fallax* and *Pleurotricha lanceolata* there is no obvious relation between the number of chromosomes of these two very similar species.

CONCLUSION.

The cytology of conjugation and division in *Pleurotricha lanceolata* has been described in detail, together with some of the cytological phenomena of encystment. The processes of division do not differ particularly from those which are known to occur among ciliates in general, with the exception of the dividing endosome and connecting intradesmose. Certain features in the division of the micronucleus are strikingly like some which occur in the mitosis of metazoan tissue cells.

A remarkable feature of encystment is the extrusion of the old macronuclei, and perhaps one of the micronuclei, through the cyst wall. After this has happened the normal nuclear complex is rebuilt from the single micronucleus remaining. Whether the presence of the latter is regularly due to the extrusion of the other micronucleus from the cell, or to the fusion of the two original micronuclei is not altogether certain.

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The cytological changes of conjugation are in general similar to those previously described in other ciliates, and especially resemble those which occur in *Oxytricha fallax*, but there are important differences in detail. Reduction is shown to occur in the second maturation division. The diploid number of chromosomes is too great to determine exactly, but is probably forty. There may be a third cleavage division in addition to the two which regularly occur, and in this respect *Pleurotricha lanceolata* differs from any other hypotrich so far described.

Conjugation appears to be not only an unnecessary part of the life cycle of this species, at least as long as environmental conditions remain favorable, but is a very dangerous event, for 92 per cent. of one hundred exconjugants died without further division, and only 1 per cent. showed any indication of an accelerated fission rate.

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EXPLANATION OF THE FIGURES.

Magnification (unless otherwise stated) × 550; spindles × 1700.

PLATE I.

Early Stages of Division.

FIG. 1. A typical vegetative individual. Occasionally individuals are found with only one micronucleus, or with three.

FIG. 2. The first stage in division. A kernspalt appears at the outer end of each macronucleus, and the beginning of a new adoral zone can be made out (a. z.).

FIG. 3. The micronuclei show an endosome, or division center, in process of division, the halves being connected by an intradesmose. The kernspalt has moved nearer the center of each macronucleus

FIG. 4. The micronuclei have become somewhat larger, and the macronuclei have become almost round. The kernspalt is almost ready to pass off the latter entirely, and the new adoral zone is more conspicuous.

FIG. 4 *A*. An elongated, faintly staining micronucleus; seen in very early division.

FIG. 4 *B*. Same as above, but a little later.

FIG. 5. The chromatin in the micronuclei has condensed into definite threads. The macronuclei have become almost completely spherical and the new adoral zone is well developed. New cirri are making their appearance in the neighborhood of both adoral zones. Enlarged figures of the micronuclei are given under 5 *A* and *B*.

FIG. 6. The anterior micronucleus, shown enlarged in Fig. 6 *A*, has formed a definite spindle.

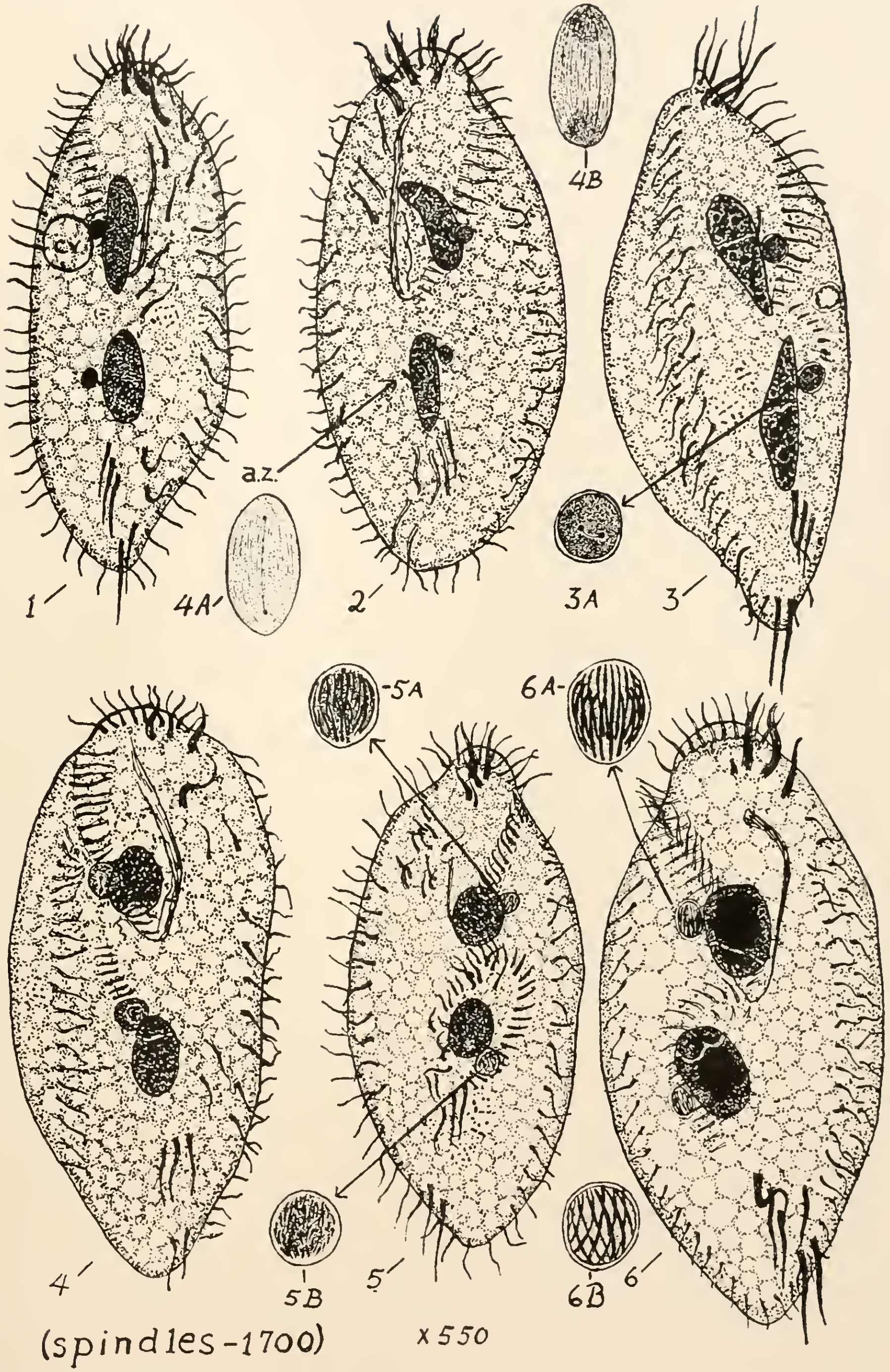
C.V.
4B
a.z.
1
4A
2
3A
3
5A
6A
4
5B
5
6B
6
(spindles-1700)
x 550

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PLATE II.

Later Stages of Division.

FIG. 7. Both micronuclei have formed spindles, which are shown enlarged in 7 *A* and 7 *B*. The cirri are larger, and the macronuclei still almost round.

FIG. 7 *C*. A spindle, magnified about 3500 times, showing the shape of the chromosomes, and the way in which they divide. The heavy strands connecting them are to be noted particularly.

FIG. 8. An individual with but one micronucleus, which is in the metaphase. The macronucleus have elongated, and the new cirri are conspicuous and large.

FIG. 9. An individual in which the micronuclei are in the anaphase.

FIG. 9 *C*. A later stage in the anaphase.

FIG. 10. A still later anaphase.

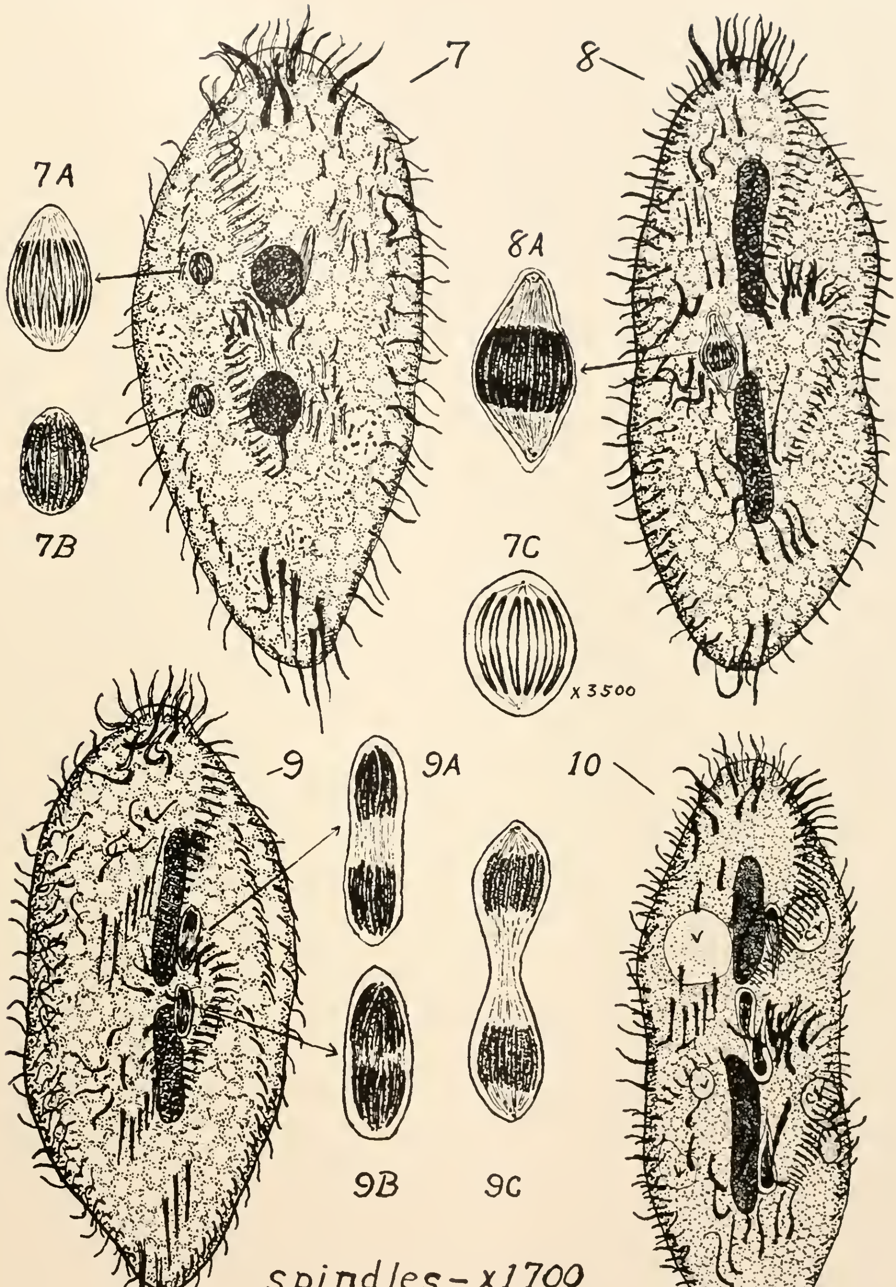
7
8
7A
8A
7B
7C
x 3500
9
9A
10
9B
9C
v
spindles - x1700
organisms - x550

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PLATE III.

Final Stages of Division; Cysts.

FIG. 11. The macronuclei are beginning to divide, and some of the old cirri have apparently disappeared. The new ones, originally formed near the adoral zones, are approaching their normal positions.

FIG. 11 *A*. A late stage in the telophase, enlarged from Fig. 11.

FIG. 11 *B*. An earlier telophase.

FIG. 12. The final stage of division.

FIG. 13. An individual which has just divided. The shape differs somewhat from that of a typical vegetative individual, and the anal cirri are not yet quite in their normal positions.

FIG. 14. An early stage of encystment, in which the individual has rounded up, but some of the cirri still remain, and the contractile vacuole is still functional. × 750.

FIG. 15. An encysted animal, in which the cyst wall has just been secreted. × 750.

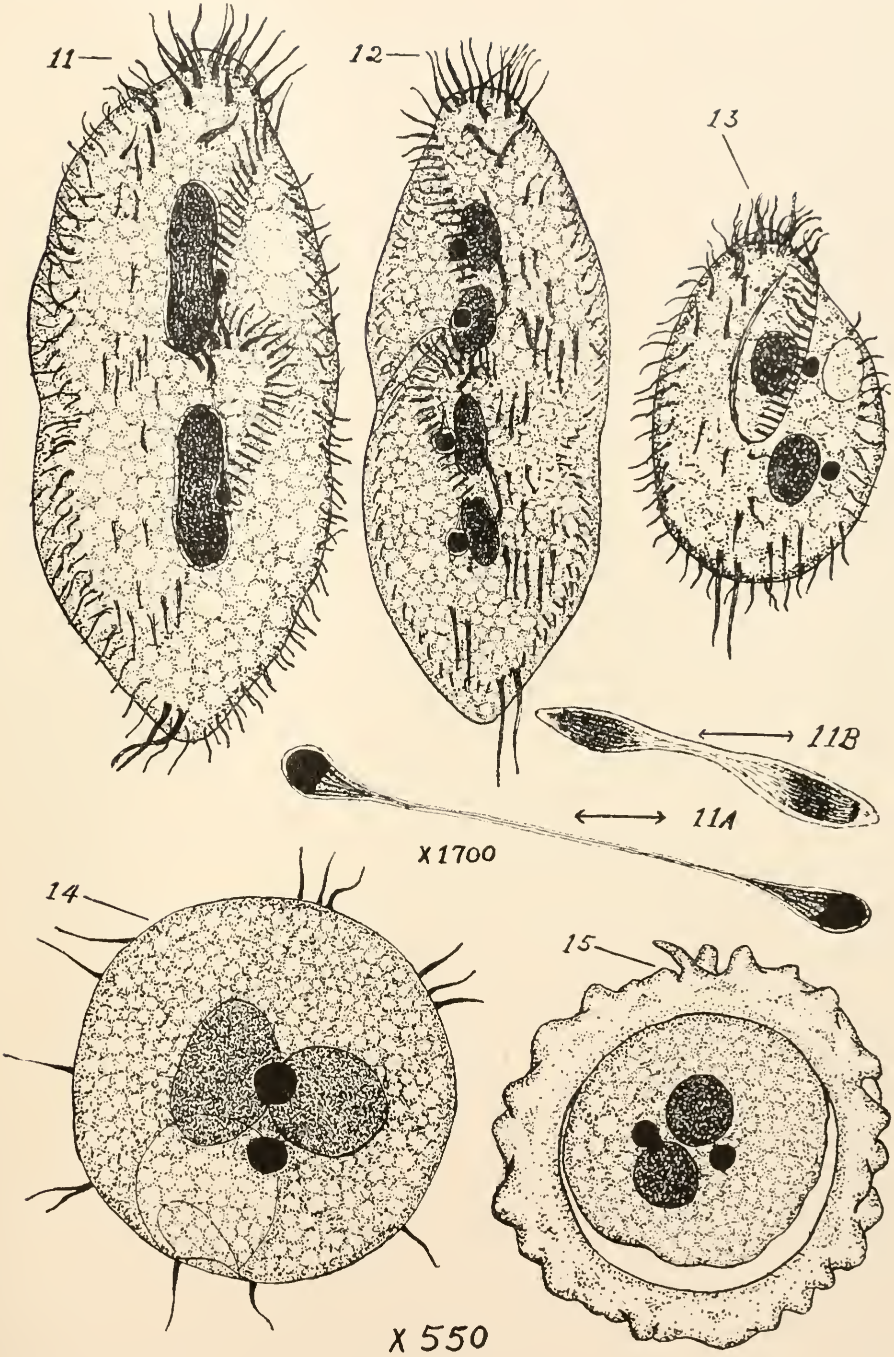

11
12
13
11B
11A
X 1700
14
15
X 550

PLATE IV.

Cysts in Various Stages.

FIG. 16. Showing the extrusion of the macronuclei and a small portion of the cytoplasm.

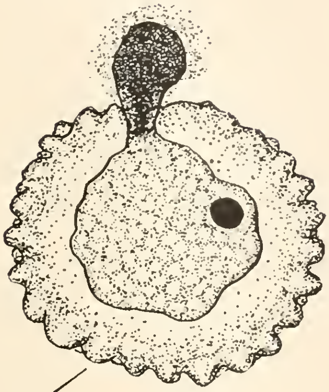
FIG. 17. An encysted animal in which only one micronucleus remains. × 750.

FIG. 18. A cyst containing three nuclei and some darkly staining material which is however not chromatin. × 750.

FIG. 19. A cyst containing seven nuclei, several of which appear to be in mitosis. × 750.

FIG. 20. A cyst in which reorganization is almost complete. × 750.

FIG. 21. Cyst containing a completely reorganized animal. × 750.



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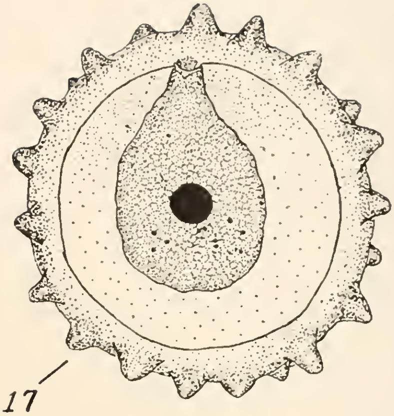

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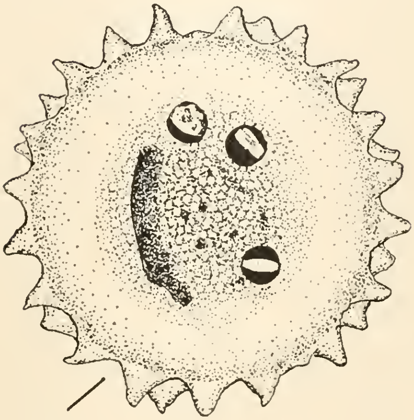

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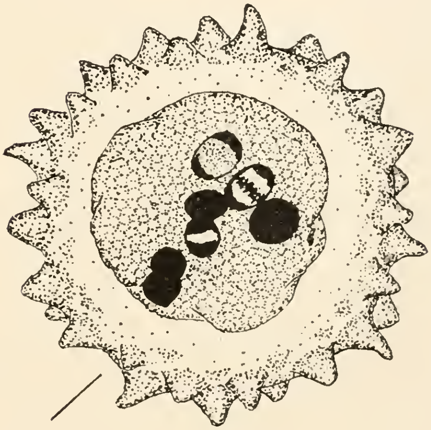
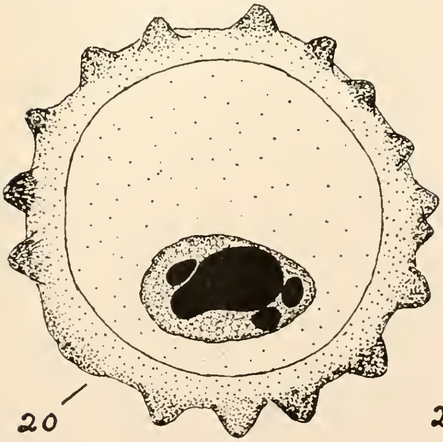


19



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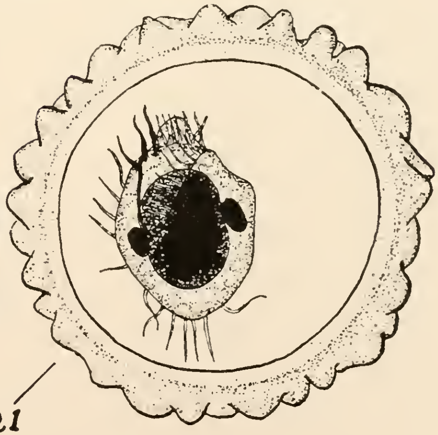

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PLATE V.

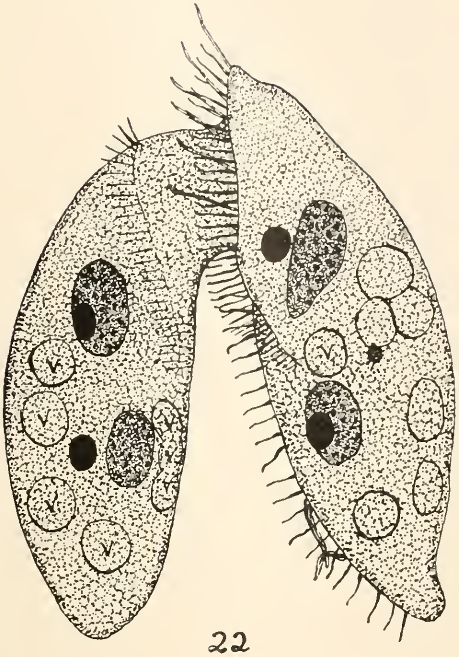
First Maturation Division.

FIG. 22. Initial stage in conjugation, showing the manner of fusion.

FIG. 23. A pair in which the micronuclei have begun to enlarge, preparatory to the first maturation division.

FIG. 24. Here the division centers have made their appearance, and in one nucleus the spindle fibers are becoming visible. Enlarged in 28 *B* and 28 *C*.

FIG. 25. Parachute stage, just prior to the formation of chromosomes. Shown enlarged in Fig. 28 *E*.



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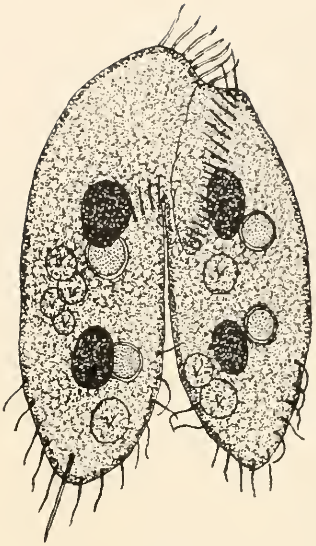

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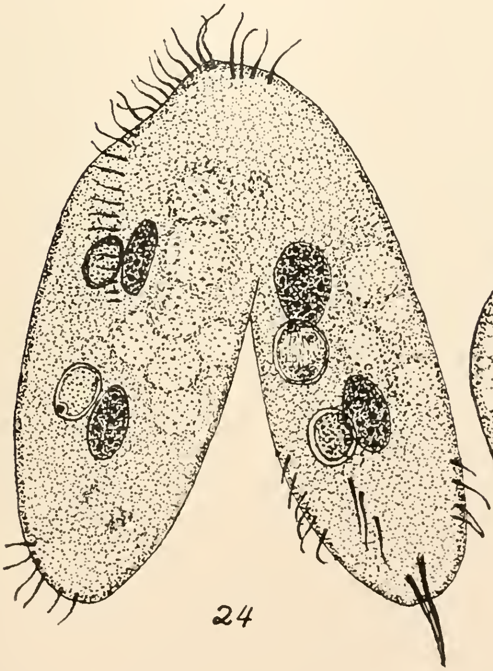

24

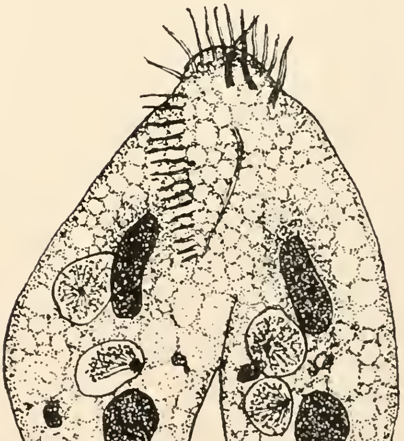

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PLATE VI.

First Maturation Division.

FIG. 26. Here the micronuclei have entered the anaphase. Two of the spindles are shown enlarged in Figs. 28 *H* and 28 *K*.

FIG. 27. A pair in which a telophase may be seen in each member.

FIG. 28 *A–M*. Various stages of the first maturation division arranged consecutively.

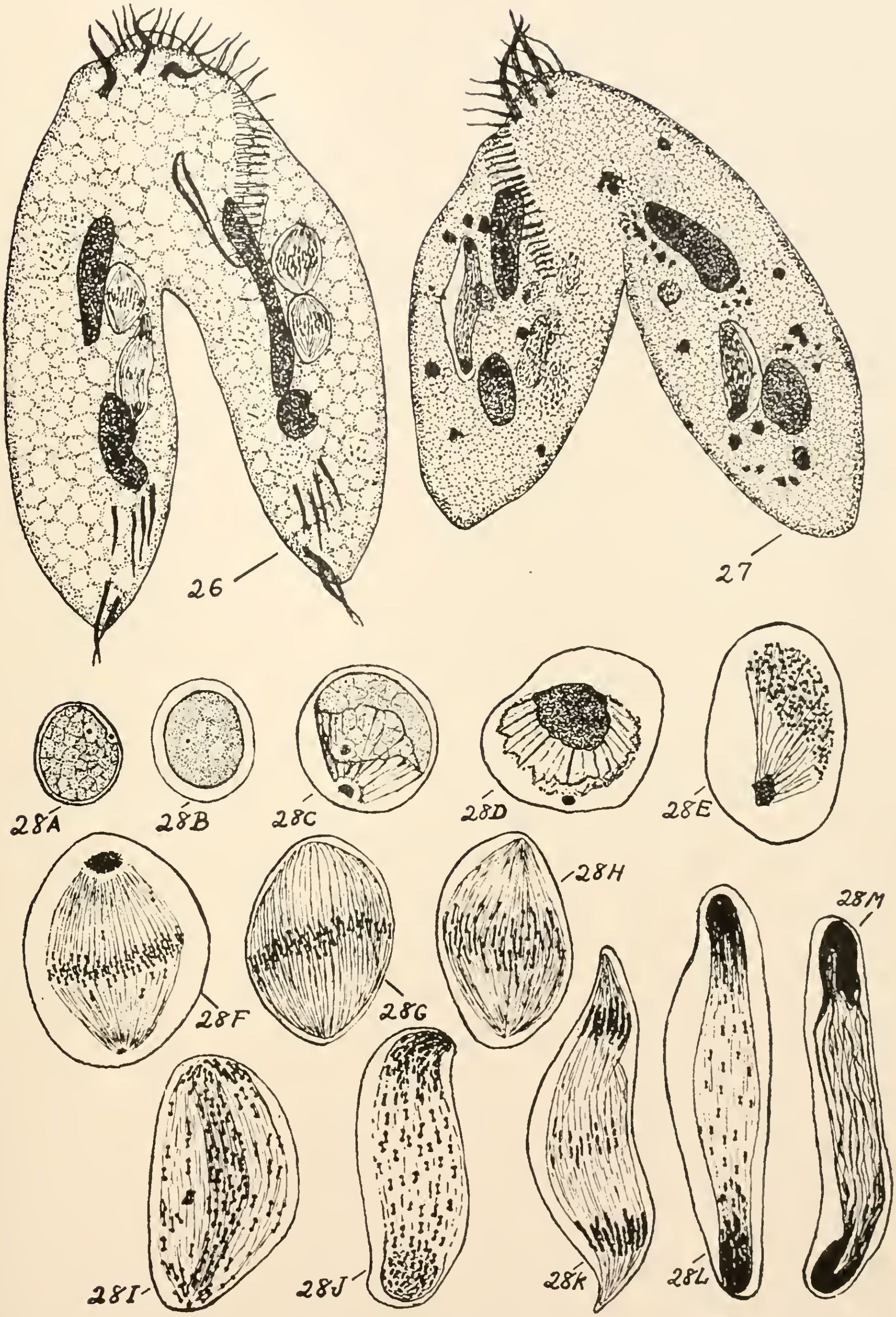
26
27
28A
28B
28C
28D
28E
28F
28G
28H
28M
28I
28J
28K
28L

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PLATE VII.

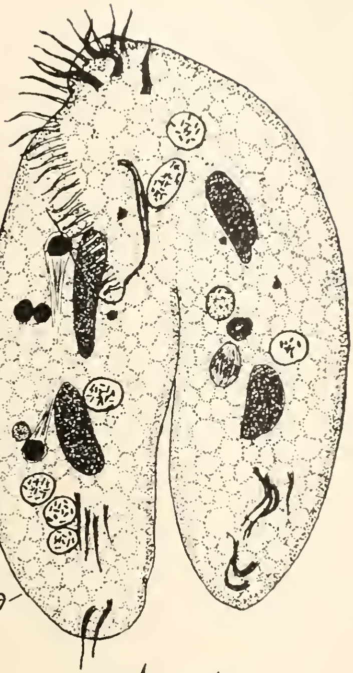
Second Maturation Division.

FIG. 29. The left-hand member of the pair shows the final stage in the telophase of the second maturation division. On the right there is a prophase and an early anaphase.

FIG. 30. Each individual of this pair shows a telophase of the second maturation division, and a prophase of the third division is also to be seen in the left-hand member.

FIG. 31. Two prophases of the second maturation division are visible in the left-hand member and a prophase of the third division may be seen on the right.

FIG. 32 *A–H*. Various stages in the second maturation division. "*A*" shows both products of the first maturation division, one of which is already in the prophase of the following division. "*C*" is also a prophase. and should probably precede "*A*."



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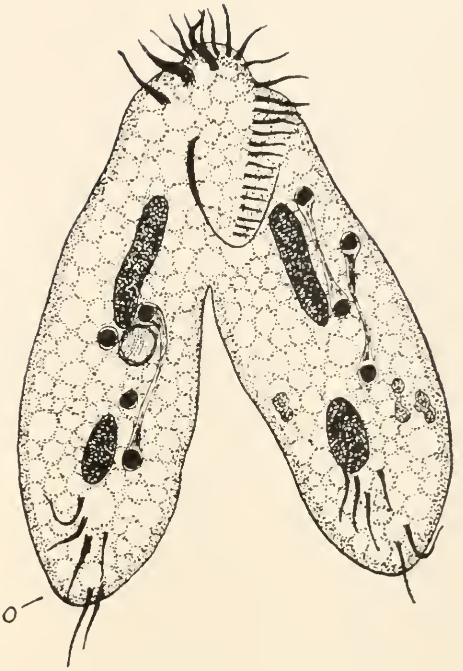

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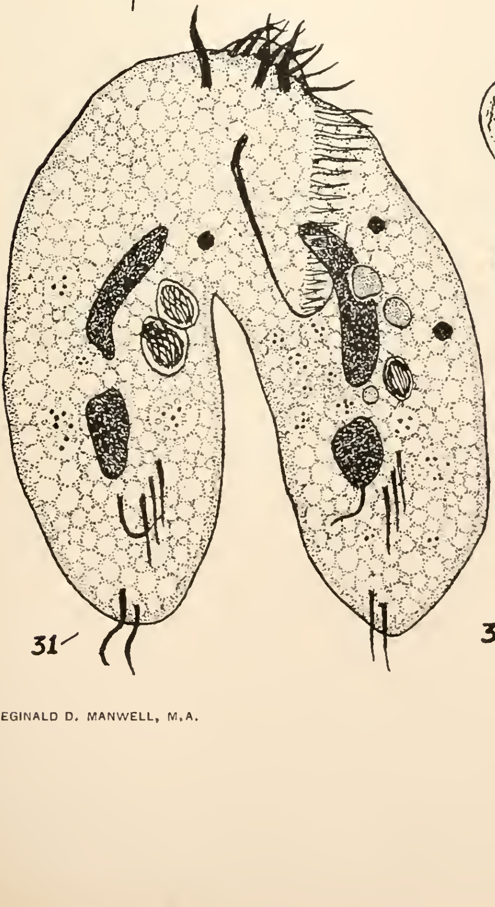

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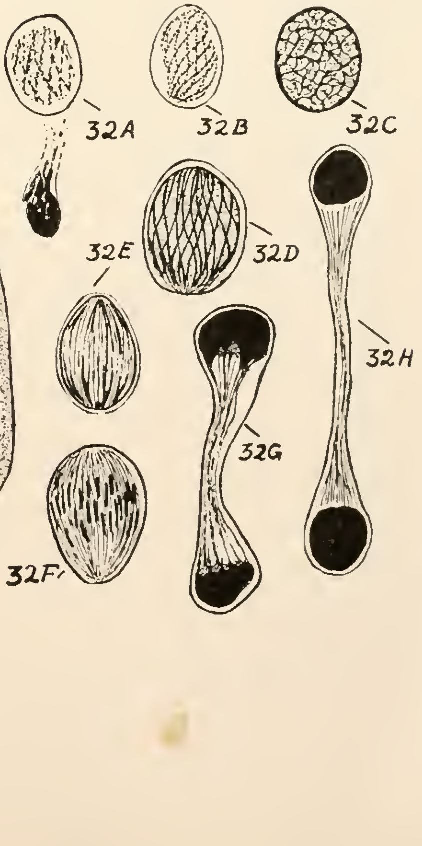
32A
32B
32C
32D
32E
32H
32G
32F

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Plate VIII.

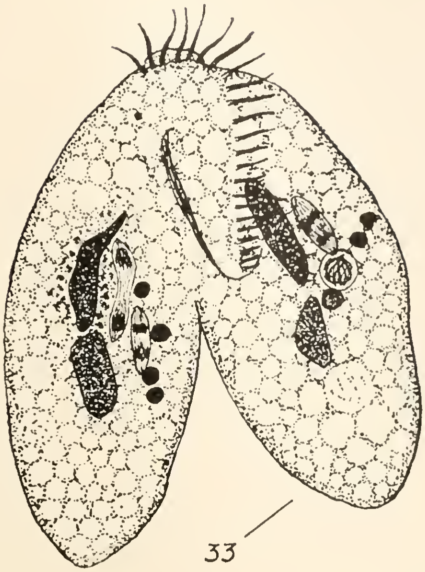
Third Maturation Division.

Fig. 33. A pair undergoing the third maturation division. Two anaphases appear on the left and a prophase and anaphase on the right.

Fig. 34. Each member of the pair shows a metaphase, and in addition there is an anaphase in the left-hand member and a prophase in the one on the right.

Fig. 35. A metaphase and anaphase appear on the left, and a metaphase and telophase on the right.

Fig. 36 *A–I*. Stages of the third division, arranged consecutively.



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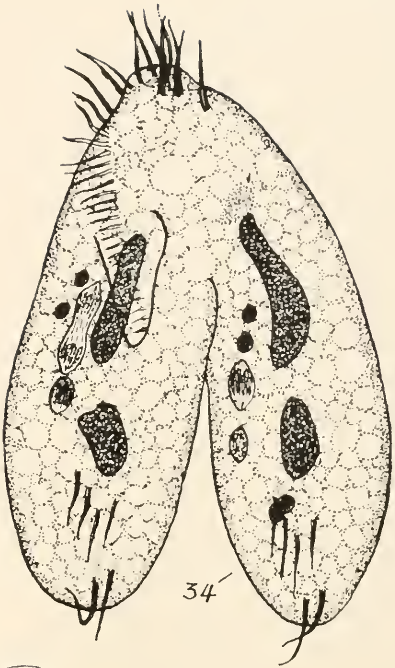

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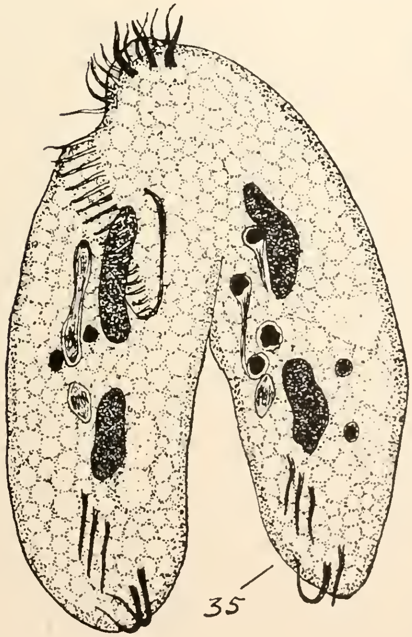

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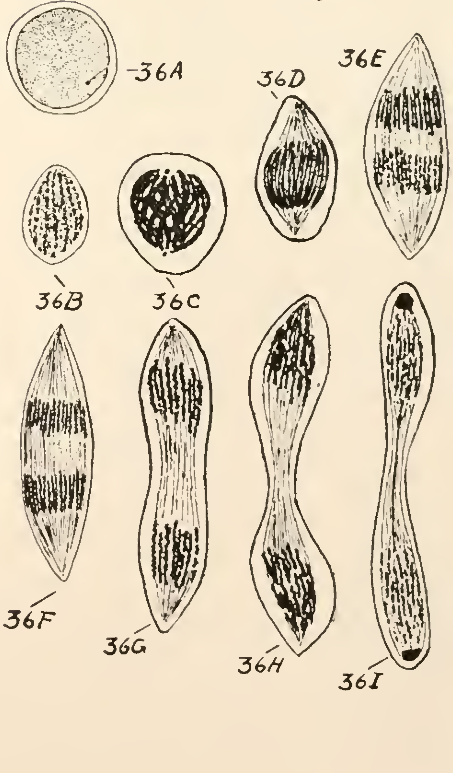
36A
36D
36E
36B
36C
36F
36G
36H
36I

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PLATE IX.

Interchange and First Cleavage.

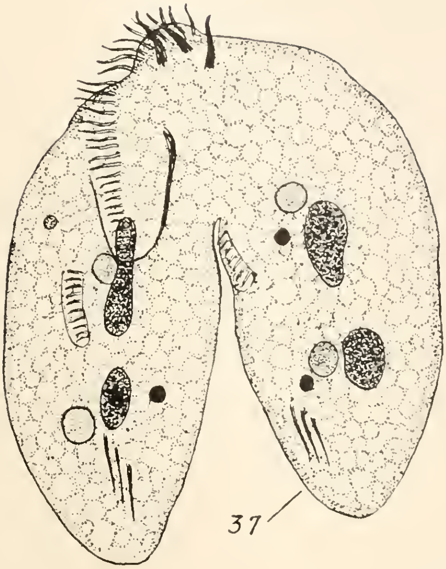
FIG. 37. The interchange. The wandering nuclei have already migrated but have not yet fused. Shown as large and relatively faintly staining bodies. New adoral zones are beginning to form.

FIG. 38. The left-hand member of this pair shows the fusion nucleus.

FIG. 39. In the left-hand individual the pronucleus has not yet entered the prophase of the first cleavage division, but division in the right-hand member is well advanced.

FIG. 40. The pronucleus of Fig. 38, enlarged × 1700.

FIG. 41 *A–H*. Stages of the first cleavage division, arranged consecutively.



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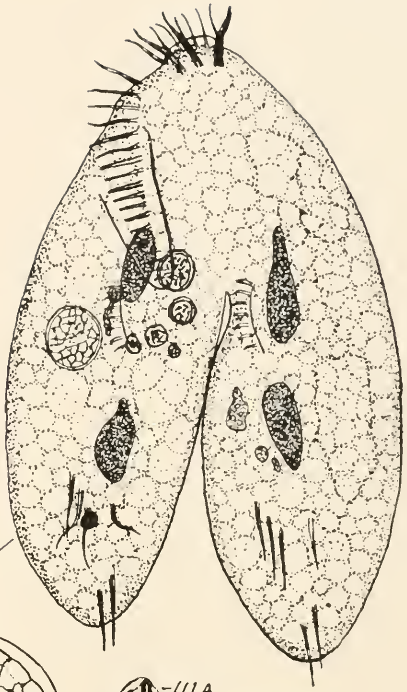

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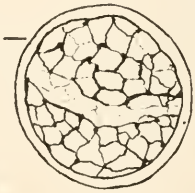

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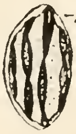
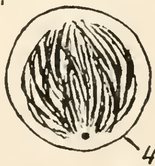


41A



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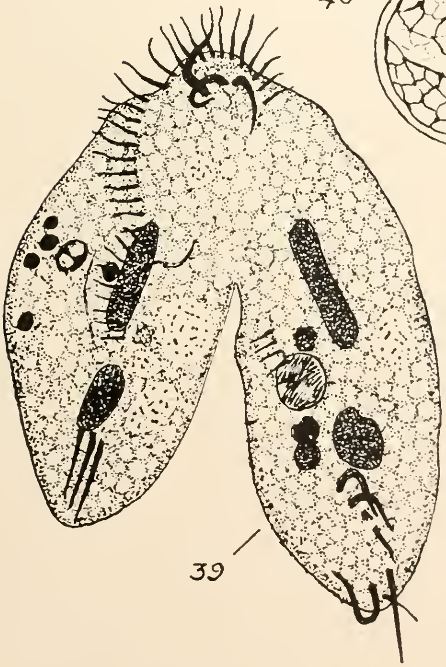

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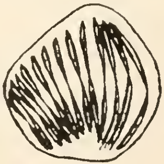
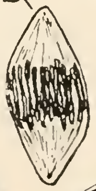
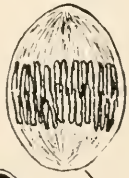


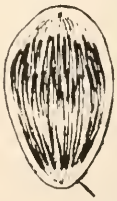
41C



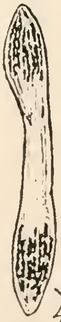
41D



41E



41F



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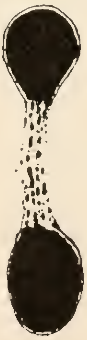


41H

PLATE X.

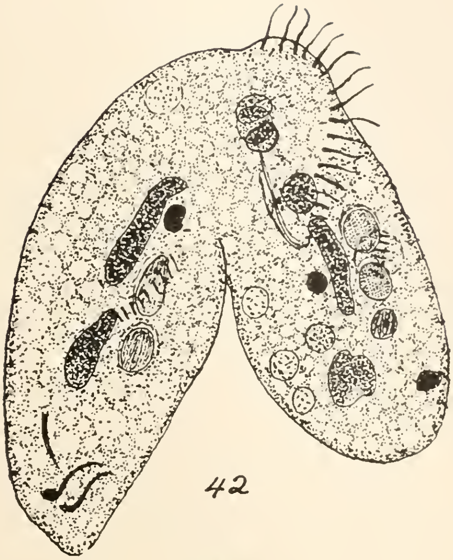
The Second Cleavage Division.

FIG. 42. Shows several prophases.

FIG. 43. In this pair a prophase and anaphase are visible in the left-hand individual, and a prophase and telophase in the right.

FIG. 44. A prophase and telophase may be seen in each of the members of this pair, and also several degenerating micronuclei which still persist from the maturation divisions.

FIG. 45. The cleavage divisions are complete in the left-hand individual but a prophase of the second division is still to be seen in the other member.



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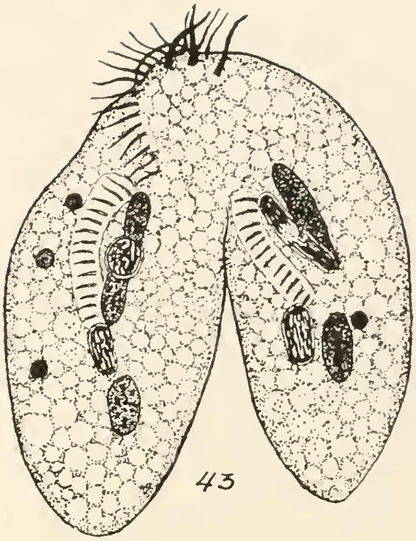

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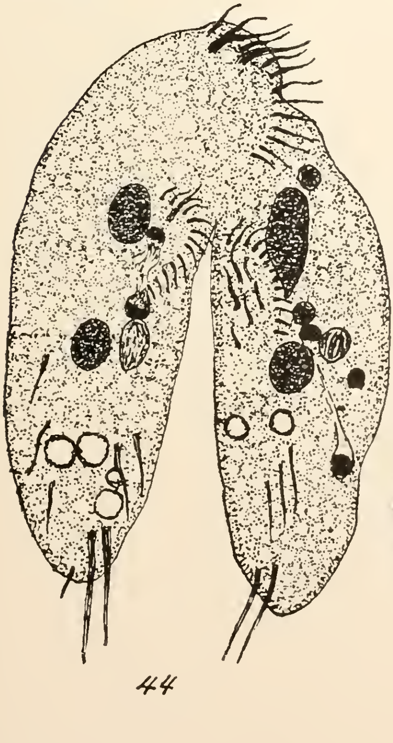

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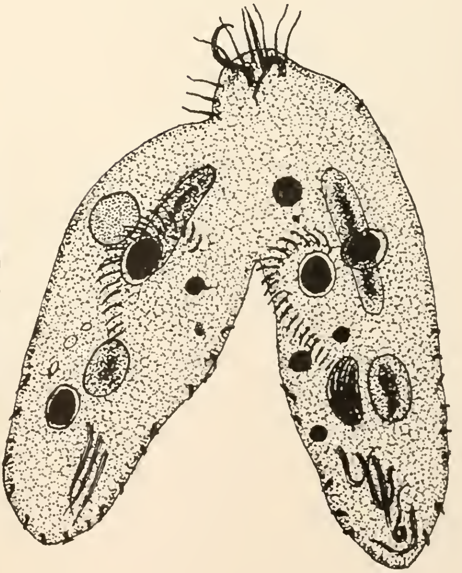

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PLATE XI.

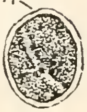
The Second Cleavage Division and Early Stages of Reorganization.

FIG. 46 *A–M*. Stages of the second cleavage division, arranged consecutively.

FIG. 47. Shortly after the conclusion of the last cleavage division. Each individual contains several micronuclei, one of which is increasing in size and will give rise to the macronuclei of the reorganized exconjugant.

FIG. 48. The stage is similar to that in the preceding figure, except that the old macronuclei are becoming vesicular and are beginning to stain much more heavily than normally.

FIG. 49. A pair more completely fused than usual, and with the distribution of micronuclei irregular. The macronuclei are noticeably degenerating.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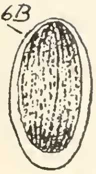
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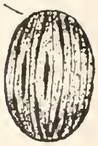
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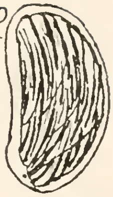
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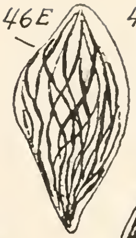
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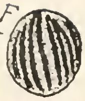
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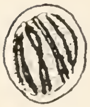
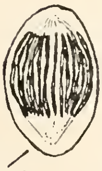
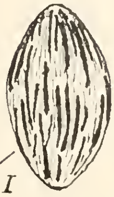

46G


46H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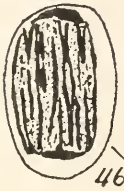
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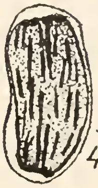
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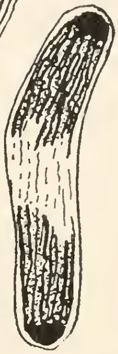
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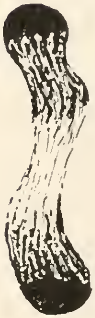
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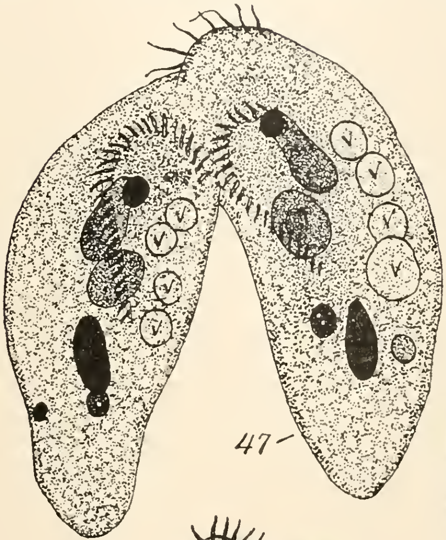
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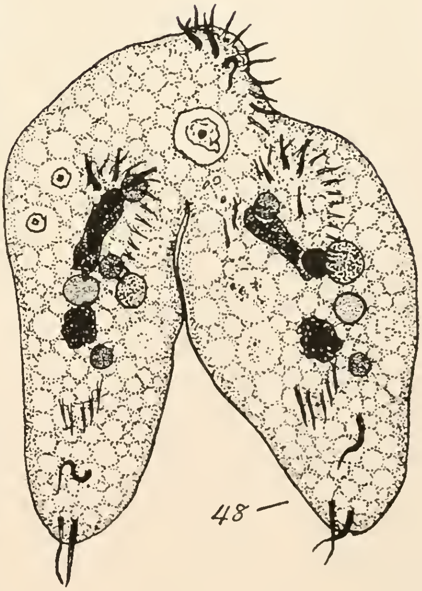
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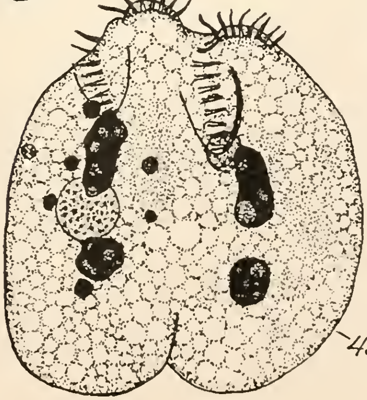
49

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PLATE XII.

Reorganization.

FIG. 50. A pair the members of which are about to separate. The fourth of the nuclei arising from the last cleavage has apparently degenerated in each individual, and the macronuclei have taken on the typical circular and vacuolated appearance which persists until their final degeneration.

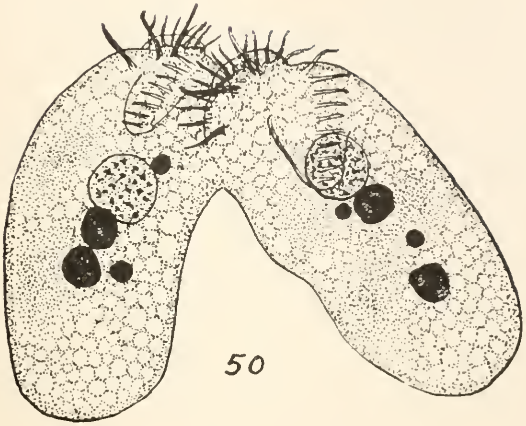
FIG. 51. Exconjugant shortly after separation. Typical of those individuals in which a third cleavage division has occurred.

FIG. 52. Exconjugant somewhat longer after separation than the one shown in Fig. 51.

FIG. 53. Exconjugant in somewhat more advanced stage than the preceding The remnants of the old macronuclei have finally disappeared.

FIG. 54. Reorganization in this individual has apparently been completed without preliminary division.

FIG. 55. One of the daughter individuals produced by the first division of an exconjugant. The macronucleus is in process of division by which the normal nuclear constitution will be finally restored.



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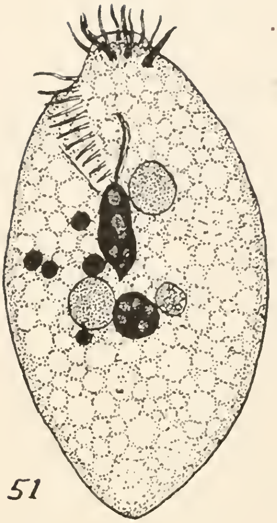

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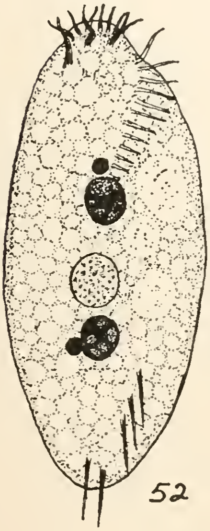

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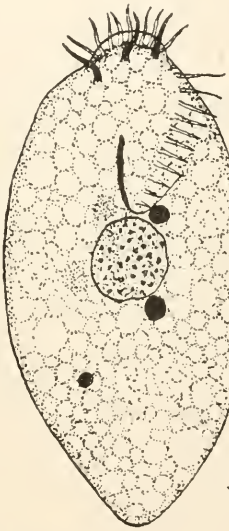

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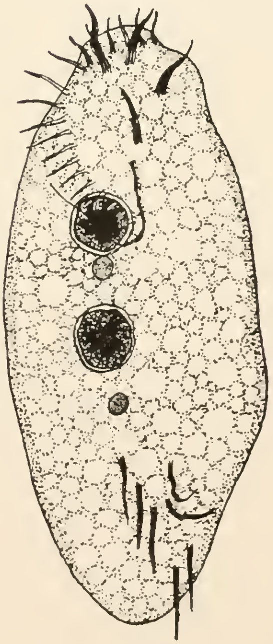

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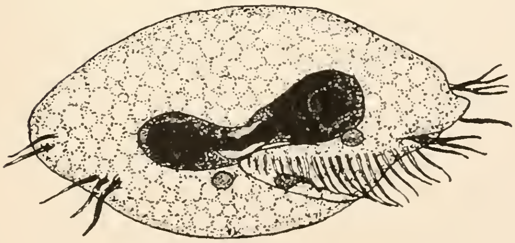

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Vol. LIV *June, 1928* *No. 6*

BIOLOGICAL BULLETIN

NODES AND CHIASMAS IN THE BIVALENTS OF *LILIUM* WITH REGARD TO SEGMENTAL INTERCHANGE.

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The bivalents of several species of *Lilium*, during the interval between the double-thread stage and the metaphase, have already been examined by a number of good observers. Hence for further fruitful work three things seem more or less necessary: á narrowly limited aim; perfect fixation, which is probably best attained by fixing the pollen-mother-cells outside the anther; and accurate microscopy, which will doubtless include the use of a small circular diaphragm on the source of light, a corrected immersion condenser, and appropriate light-filters.

To attain uniform terminology, the writer ventures to use in this paper the terms, diaphase, instead of diakinesis; diplophase, for diplotene stage; etc.

Bulbs of *Lilium longiflorum* (clonal variety, Easter lily) were potted late in the year, in a cool greenhouse. Flower-buds somewhat less than two centimeters long usually showed the required stages of the pollen-mother-cells; namely, late diplophase, diaphase (early, middle and late), and metaphase. One anther at a time could be removed, so that six stages at intervals of an hour or two could be prepared from each bud. The anther was cut up, and the short segments squeezed out in a drop of iron-acetocarmine. (Spreading out the pollen-mother-cells on a slide, fixing with Flemming's solution, and staining with iron-hæmatoxylin or iron-brazilin, gave preparations less suited for the purpose.) The thin layer of liquid was allowed to evaporate under a large coverglass for several hours, until the cells were flattened by the capillary pressure. Then the edges were sealed (by a thick solution of dammar in xylol, or better) with melted paraffin wax. For oil-immersion objectives the tube-length required to be slightly increased, according to how many microns the chromosomes extended below the coverglass. The apochromatic objective for water immersion, of 1.25 aperture, and the binocular microscope, were mainly used.

Flower buds of *L. regale,* from the greenhouse, gave nearly as clear figures as those of *L. longiflorum.* Buds from *L. candidum,* grown in the open, in June, gave excellent metaphases. But in late July and August, buds of *L. tigrinum, L. speciosum* and *L. auratum* had granular pollen-mother-cells; and though these cleared up in time in acetocarmine, yet they were not so clear at diplophase and diaphase as corresponding stages of *L. longiflorum.* At anaphase also, the chromosomes were shorter and thicker in the plants grown in the summer than in those species raised in the greenhouse in February.

An examination of the chromosomes of *L. longiflorum,* at the anaphase of the first division, shows that there are two large J chromosomes; one with the constriction perhaps one-third from one end, and the other with the constriction near one-quarter from the end. The remaining ten chromosomes have the primary constriction more or less near to, or at, the end; so that the separated segment is small or invisible at this stage. (One of these chromosomes shows a secondary constriction.)

The leptophase and zygophase closely resemble Newton's figures for *Tulipa* (Newton, 1927). In the late diplophase, before the bivalents are separate enough to be countable, nodes may be observed in the double thread. At the earliest diaphase, as soon as separate bivalents can be distinguished, many nodes are visible. It is difficult to find a cell, however, in which all twelve bivalents are free enough from overlap to permit the counting of all the nodes. However, Fig. 1 is a camera drawing of a cell showing the 12 bivalents at the early diaphase. There is here a total of 39 nodes; made up by one bivalent with 5 nodes, four with 4 nodes, four with 3 nodes, and three with 2 nodes. Other cells at the same stage seemed to have about the same numbers of nodes, though all 12 bivalents could not be counted. In *L. regale,* also, this stage seems to have a larger number of nodes than the following stages; as has, indeed, been noted by previous observers in both plants and animals. In *Tulipa*, which is allied to *Lilium*, Newton's drawings allow the estimation of about 34 nodes in 11 of the 12 bivalents at the latest diplophase or earliest diaphase. Here, there seem to have been one bivalent with 5 nodes, one with 4, seven with 3, and two with 2 nodes.

Between the late diplophase and the late diaphase, many of these nodes disappear. At middle diaphase, so far as incomplete counts have been made, the numbers are less than in early diaphase and more than in late diaphase. At late diaphase, the numbers of nodes in five cells of *Lilium longiflorum,* where all 12 bivalents were widely spaced and the nodes readily counted, were 24, 24, 23, 21, and 21, respectively. The average of these is 22.6. Fig. 2 is a camera drawing of one of these cells. In *Tulipa,* Newton's drawing shows about 21 nodes at late diaphase. Thus in Lilium we have a diminution in the number of nodes, from the earliest diaphase to late diaphase, of 43 per cent.; while in *Tulipa* the estimated loss is also slightly over 40 per cent. It is possible that if the counts could have been made earlier in the diplophase the loss would have been shown to be somewhat larger. Hence we may say that, in *Lilium* and *Tulipa,* at least, somewhat less than half of the original nodes disappear before the metaphase.

At the metaphase, the numbers of nodes were about the same as in late diaphase; namely, 24 in one metaphase of *L. longiflorum* (Fig. 3), and 21 and 20 in two metaphases of *L. regale;* averaging 21.7, as compared with 22.6 for the late diaphase. In these cases the metaphase chromosomes were squeezed from the cells, and cases were readily procured in which the nodes of all 12 bivalents could be accurately counted.

The nodes at late diaphase and at metaphase are doubtless chiasmas (Janssens, 1924), as they are also in *Uvularia, Hyacinthus,* and *Allium* (Chodat, 1925). A chiasma can be distinguished microscopically by the following points. (1) When the bivalent is normally flat, or is flattened by pressure, two chromatids may be seen to pass on, and two to form an X. Thus Fig. 4 shows a metaphase bivalent of *L. candidum,* slightly compressed, at three different levels, the focus of the microscope descending from left to right. There are two chiasmas visible, of which the left one is the clearer. Fig. 5 shows another flattened bivalent from the same metaphase plate as Fig. 4, drawn as usual with shifting focus. Here the two chiasmas are clearly alternating with regard to the chromatids. (2) In a chiasma at the diplophase or diaphase, the cross junction can often be seen to be thinner than the continuous threads. In a half turn of a spiral, the cross junction would of course be of double thickness. (3) When sufficient pressure is applied to the diaphase bivalents, after being some days in acetocarmine, they seem to break across only at the nodes. The separation of the strands in a chiasma would doubtless make it a point of weakness. (4) At late diaphase and metaphase, the portions of the bivalents on each side of a chiasma are in planes more or less at right angles to each other (Fig. 3). (5) At the first anaphase, the constituent chromatids do not separate in the halves of vertical V's and rings, while they do in horizontal V's or rings. Fig. 6 shows this separation in *L. longiflorum*.

The nodes (chiasmas) have been counted in 96 bivalents of *Lilium*, from eight different cells, at late diaphase and metaphase. Of these, 31 bivalents had one node, 49 had two nodes, and 16 showed three nodes. Calculating the points of segmental interchange, or points of genetic crossing over, in the resulting chromosomes (four from each bivalent), we find: 119 chromosomes with no point of interchange; 184, with one point of interchange; 73, with two points of interchange; and 8 with three points of interchange. In percentages these are: 31, with none; 48, with one; 19, with two; and 2, with three points of interchange. These figures agree fairly with the figures for the four large bivalents of *Hyacinthus* (Belling, 1927), and perhaps also with the genetical results in species of *Drosophila*.

The nodes which disappear between diplophase and late diaphase do not seem to be all or mainly twists. Clearly recognizable twists (half turns of a spiral) are apparently rare in *Lilium longiflorum* and *L. regale*. Nor do these vanishing nodes seem to be chiasmas which open out; for if so, this process should have been visible, as it is at early anaphase. Their nature awaits further investigation. However, the drawings of early observers, which show bivalents in *Lilium*, or plants allied to *Lilium*, forming, at the late diplophase or early diaphase, regular right-handed or left-handed spirals of several turns, do not correspond to what is to be observed in *Lilium longiflorum*, with perfect fixation, yellow-green light, a nine-tenths condenser cone, and apochromatic oil-immersion objectives of 1.3 and 1.4 aperture.

Summary.—(1) The bivalents of six species of *Lilium* were studied, from the double-thread stage to the early first anaphase, in pollen-mother-cells fixed in iron-acetocarmine. Thirty-nine nodes were found at late diplophase or early diaphase.

(2) In 5 late diaphases, the average number of nodes was nearly 23, and in 3 metaphases the average number was nearly 22. Thus there was a loss of 43 per cent. of the nodes between late diplophase and late diaphase.

(3) Out of 96 bivalents, there were 31 with one node, 49 with two nodes, and 16 with three nodes. This would result in chromosomes having 31 per cent. with no point of segmental interchange, 48 per cent. with one point, 19 per cent. with two points, and 2 per cent. with three points of segmental interchange.

(4) Dixon's term, "strepsitene," seems to have been a misnomer in the case of *Lilium*. Fifty-seven per cent. of the nodes were demonstrably chiasmas.

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EXPLANATION OF PLATE I.

Fig. 1. Early diaphase in a pollen-mother-cell of *Lilium longiflorum*. Camera drawing from an iron-acetocarmine preparation (as were also the other five figures). Cytoplasm and chromosomes squeezed from the cell-wall, and flattened on the coverglass. Viewed with the apochromatic oil-immersion objective 60, of 1.3 aperture, used with the binocular attachment. Tube-length increased to correct for watery layer. Wratten film, No. 66, and water-immersion condenser giving aperture of 1.2, used. There were 39 nodes counted. Some of them were seen to be chiasmas, but the detail was too fine to be shown in this drawing. Only one was proved to be a half twist.

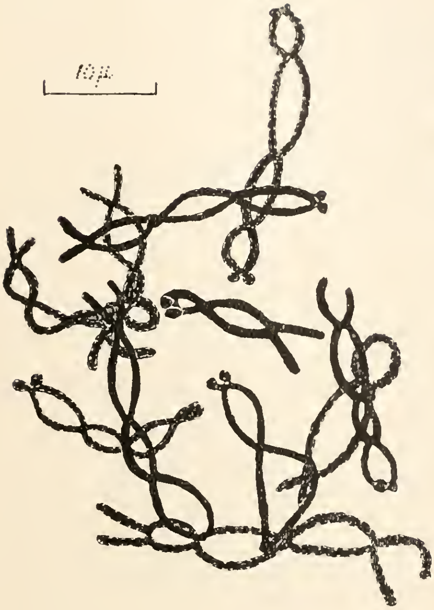
Fig. 2. Late diaphase of *Lilium longiflorum*. Preparation and optical apparatus as in Figure 1, except that lower eyepieces were used. There were 24 nodes. Many could be seen to be chiasmas. Probably all were chiasmas. Stages like this could be had in abundance, and the nodes could be easily counted.

Fig. 3. Metaphase bivalents of *Lilium longiflorum*. Magnification, etc., as in Figure 2. The nodes can be counted with accuracy at this stage, and can be shown to be chiasmas. There were 23 nodes. This stage is common.

Fig. 4. Metaphase bivalent of *Lilium candidum* which has been squeezed flat by due pressure, drawn in three focal planes. Viewed with apochromatic water-immersion objective 70. Description in text.

Fig. 5. Another bivalent from the same cell as that drawn in Fig. 4. Camera drawing with shifting focus of microscope. Described in the text.

Fig. 6. Separation of the homologues in the metaphase and early anaphase of *Lilium longiflorum*. The first four above show stages in the separation of the two larger bivalents. The two below, and the one on the right are shorter bivalents. Chiasmas are evident.



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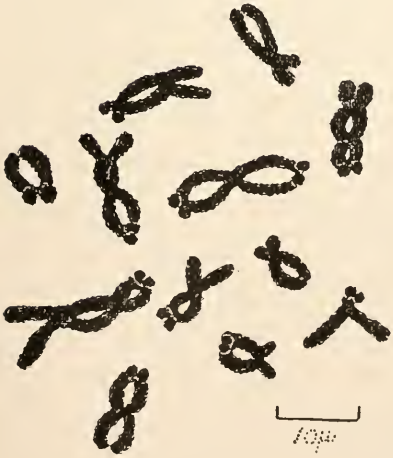

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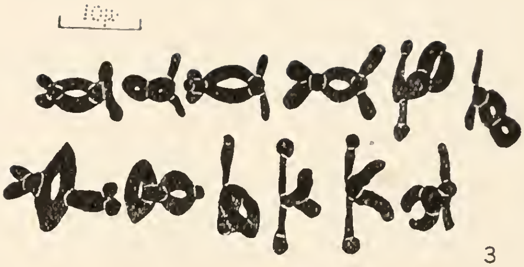

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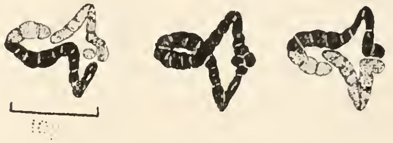

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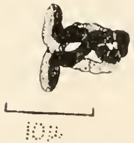

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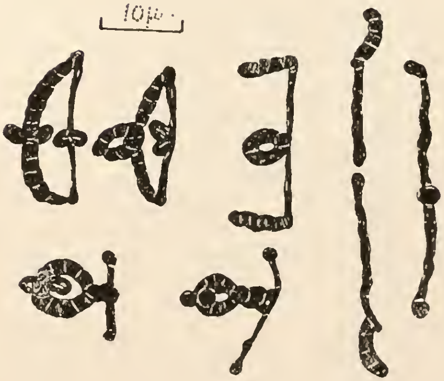

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THE NEUROMOTOR APPARATUS OF *CHLAMYDODON* SP.

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INTRODUCTION.

While working on the life cycle of *Chlamydodon,* a system of fibers around the mouth was found, and, on account of the relation of these fibers to the pharyngeal basket and other organelles, it seemed worth while to make a further study.

As most of the literature on the subject of conductile fibers, especially in the Protozoa, has been reviewed by Taylor (1920), Rees (1922), and Calkins (1926), it seems unnecessary to take up the subject here.

Kofoid applied the term Neuromotor Apparatus to the complex fibrillar system associated with blepharoplasts, parabasal bodies, etc., found in *Giardia.* Other systems of this kind have been described for the flagellates by other workers.

In the ciliates Sharpe (1915) was the first to give the term Neuromotor Apparatus to a complex system of fibrils, having a center or motorium, which he found in *Diplodinium ecaudatum.* Since that time, similar systems have been described for seven other ciliates as follows: Yocom (1913) in *Euplotes patella;* MacDonald (1922) in *Balantidium coli* and *suis;* Rees (1922), *Paramecium caudatum;* Visscher (1925), *Dileptus gigas;* Campbell (1926 and 1927), *Tintinnopsis nucula* and *Favella;* and Picard (1927) *Boveria teredinidi. Euplotes* represents a highly specialized type of Neuromotor Apparatus, *Paramecium caudatum* a generalized type, while that of *Chlamydodon* represents a combination of the two types.

ACKNOWLEDGMENTS.

The work was begun at the Marine Biological Laboratory and completed at Agnes Scott College. I wish to thank Dr. Gary N.

Calkins, of Columbia University and the Marine Biological Laboratory, for advice and helpful criticism during the progress of the work, and Miss Ruth B. Howland for help that made the work of microdissection possible.

Material and Methods.

The animals used in this investigation were collected from Knowlton's ditch at Woods Hole, Mass. A small portion of algae, chiefly Oscillatoria, from the same ditch, placed in a syracuse dish in Knop's solution one part, distilled water ten parts, furnished an excellent culture medium. By making sub-cultures, the animals were kept in the laboratory at Agnes Scott College for two winters.

Various killing and fixing solutions were used, the best results being obtained with Schaudinn's Fluid, Bouin and strong Flemming. If the material was to be stained with Mallory's connective tissue stain, it was fixed with Zenker's or Picro-mercuric fluid, using the time schedules recommended by Sharp and Yocom. Some of the best preparations were obtained by the use of .3 per cent. hæmotoxylin, long method.

Two methods of embedding were used: (1) by the aid of a LeFevre embedding dish, and (2), by killing a whole culture in a Syracuse dish, and embedding small bits of algæ to which the animals were attached. This was found to be the most satisfactory method, as there was little danger of losing the animals, and the material could be quickly handled; also it gave an abundance of material. Sections were cut from 2–4μ thick. On account of the thickness of *Chlamydodon,* it is impossible to work out the system of fibrils in whole mounts.

All drawings were made with the camera lucida, except Fig. 15, and with the use of 1/7 Leitz oil immersion lens and 12 × oculars. Details were worked out with 12 × oculars and a Zeiss 1.5 mm. apochromatic lens. A 250-watt light in a Zeiss microscope lamp was used for illumination.

The Genus *Chlamidodon.*

The genus *Chlamidodon* was named and described by Ehrenberg in the *Proceedings of the Berlin Academy* in 1835. He states that he discovered *Chlamidodon mnemosyne* in the waters of the Baltic Sea near Wismar, Aug. 26, 1834. In 1838, in his Infusionsthierchen als volkomene Organismen, he mentions this form again, and, in addition to the "teeth apparatus" mentioned in his first paper, he describes, a "colorless oval shield," projecting on all sides beyond the body, and covering its body. Stein (1859) differs with Ehrenberg in some details, *e.g.*, he says that he found only eight instead of sixteen trichites in the basket, and he thinks the "oval shield" is just part of the body. Entz (1884) describes *Chlamydodon cyclops* Entz as having fifteen and sixteen trichites in the pharyngeal basket, while Erlanger (1890) figures *Chlamydodon mnemosyne* with sixteen. Stein changed the spelling from *Clamidodon* to *Chlamydodon.*

Ehrenberg placed the genus in the family "Euplota"; Stein placed it in the family "Chlamydodonta."

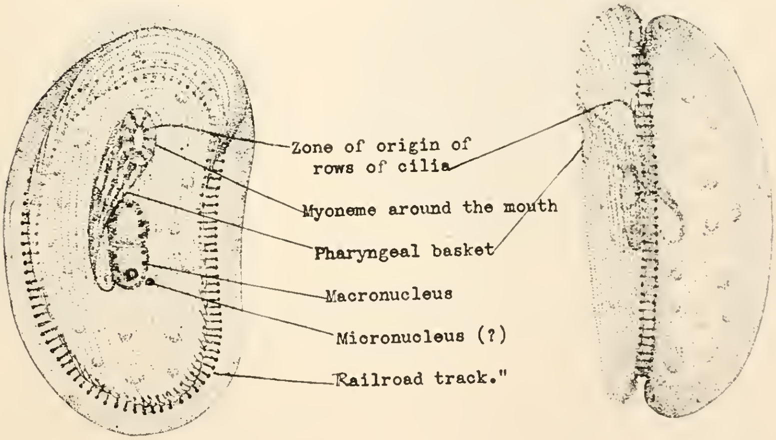
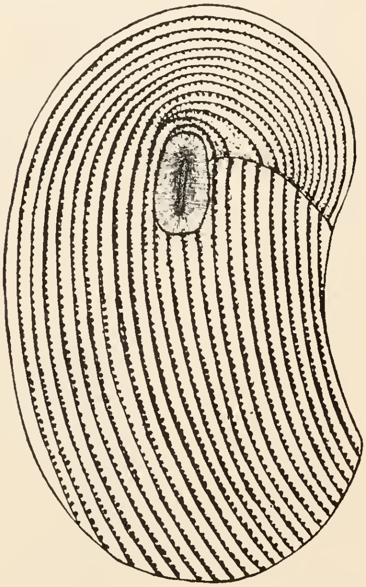


FIG. 1 *A*. *Chlamydodon* sp. (Probably a variation of *mnemosyne.*) Ventral view.

FIG. 1 *B*. Side view.

The form most common at Woods Hole is probably a variation of *Chlamydodon mnemosyne,* though it differs very much from Erlanger's description of that species. There is another species of *Chlamydodon,* found at Woods Hole, which the writer has seen only twice, and only isolated specimens. It has a very short pharyngeal basket situated at the extreme anterior end. It differs from all described species in the character of its cilia.

The species which was used as a basis for the present study, Fig. 1, measures from 60–70μ in length, and 40–45μ in breadth. Like all of the species, it is ciliated on the ventral side only, the anterior cilia being much longer than those on the other parts of the body. The cilia are very fine, and arranged in rows set close together. To get the true arrangement of cilia, the ventral surface has to be removed and flattened out. This is possible only when the "railroad track" has been cut, as this structure bounds the rows of cilia, and, at the anterior end pulls a part of the ventral surface over on the dorsal side, Fig. 1 *B* and Fig. 4. The anterior cilia take their origin from a zone on the right-hand side (ventral side up), extend around the anterior end in a half circle, thence to the posterior boundary of the "railroad track" in a somewhat curved line, Fig. 2. The cilia below the zone on the right-hand



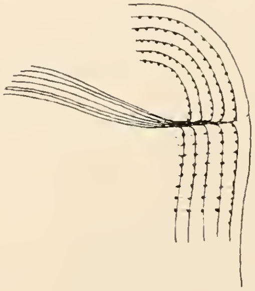
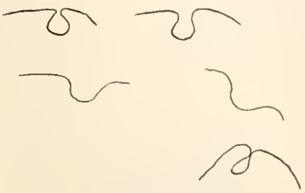


FIG. 2 *A*. Arrangement of cilia and fibers traversing the circular myoneme.

FIG. 2 *B*. Fan of fibers from cilia just before they join motorium. Delicate cross fibers from basal bodies of cilia.

side, take their origin as said zone, and extend in curved lines to the posterior boundary of the railroad track. The three central rows begin at the posterior end of the myoneme surrounding the mouth, and extend to the posterior end of the "railroad track" in the same manner as the other rows, Fig. 2. Fig. 1 *B* shows the manner in which the "railroad track" bounds the rows of cilia.



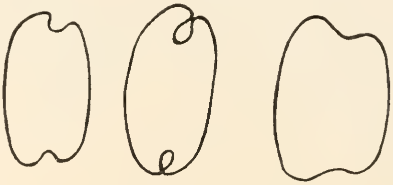


FIG. 3. Sections showing various changes in shape of the "railroad track."

FIG. 4. Sections of entire animal showing changes in the shape of the "railroad track," and changes in the shape of the animal.

The peciliar organelle known as the "railroad track" is a band of trichites encircling the animal in the manner shown in Fig. 1 *B*. In some of the older papers, the exact location of this band is a matter of discussion. We were able to pierce the dorsal wall of the animal with a micro pipette, and blow out the cell contents, including the pharyngeal basket; nothing was left except the body wall, almost entire, and the "railroad track." Its position was unchanged. It is covered with a thin pellicle, and fastened tightly to it. With the micro dissection needles, the track was cut out and pulled apart. While it was easier to tear apart where it was thin, we could not duplicate the effect shown by Erlanger. The figure in Erlanger's paper seems to show the parts of the "railroad track" easily separable into round masses of protoplasm, each having a trichite in the center.

If the animal is viewed from the ventral side, the structure looks flat, and the trichites arranged after the manner of crossties, hence the name "railroad track." A side view of whole mounts, easily obtainable, and sections, shows that the ordinary shape of this structure is half a circle. The organelle has the power of changing its shape. This is readily shown in sections; it may be almost completely closed, or wide open, Figs. 3 and 4. After this observation was made, a careful study of the living animal showed that when the lip was bent back very far the structure was closed, forming a complete ring. The anterior end was closed during ingestion of food.

The ends of the trichites seem to be heavier than the middle portion, and between each two trichites there is a thin portion with a place in the center easily pulled apart. When the organelle is cut and straightened out, it is seen that the trichite is thickened and the thin portion reduced, the end view reminding one of an accordion, Fig. 17.

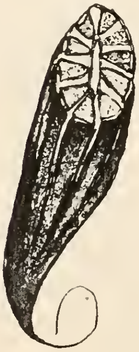


FIG. 5. The pharyngeal basket of *Chlamydodon* sp., with circular myoneme which covers it removed.

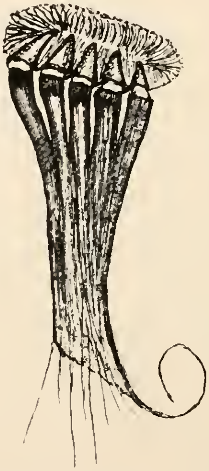
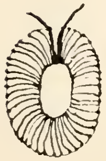


FIG. 6. Side view, showing relation of basket to myoneme.

The band or "railroad track" is not interrupted, as shown in Erlanger's figures of *Chlamydodon mnemosyne*. Just before division, a break near the center may be observed.

The pharyngeal basket is very large in proportion to the size of the animal. It is very different from the basket described by Erlanger, and figured in Doflein, p. 54. There are ten heavy trichites, the anterior ends being expanded, and the trichites showing distinctly. About half way down, the trichites seem to fuse, their identity becoming lost as the basket narrows, resembling *Chilodon uncinatus* in this respect. The posterior end has a small filament wound to the right (ventral view). At the anterior end, each trichite has a sort of cap fitted to it somewhat after the manner of a hinge, Figs. 5 and 6, and extending to the mouth opening. These caps are in the shape of a triangle, and form a lid. This lid is, in turn, covered by a circular myoneme, like a sphincter muscle, in the center of which is the mouth opening. As Figs. 6 and 8 show, the edges of the mouth opening in this myoneme fit under the points of the triangles. It is suggested that the triangles are pulled back by the myoneme, and so opens the mouth.



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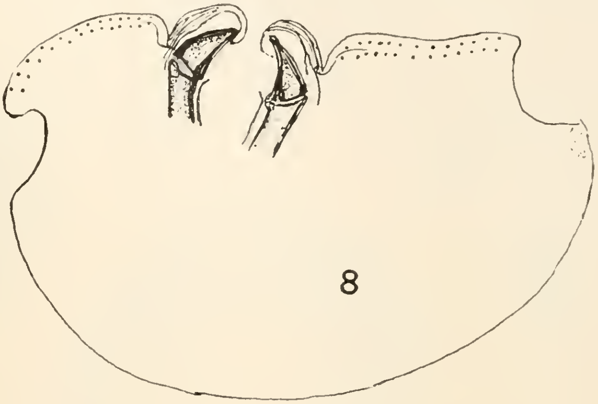

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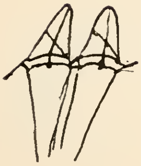

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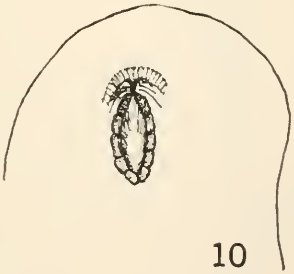

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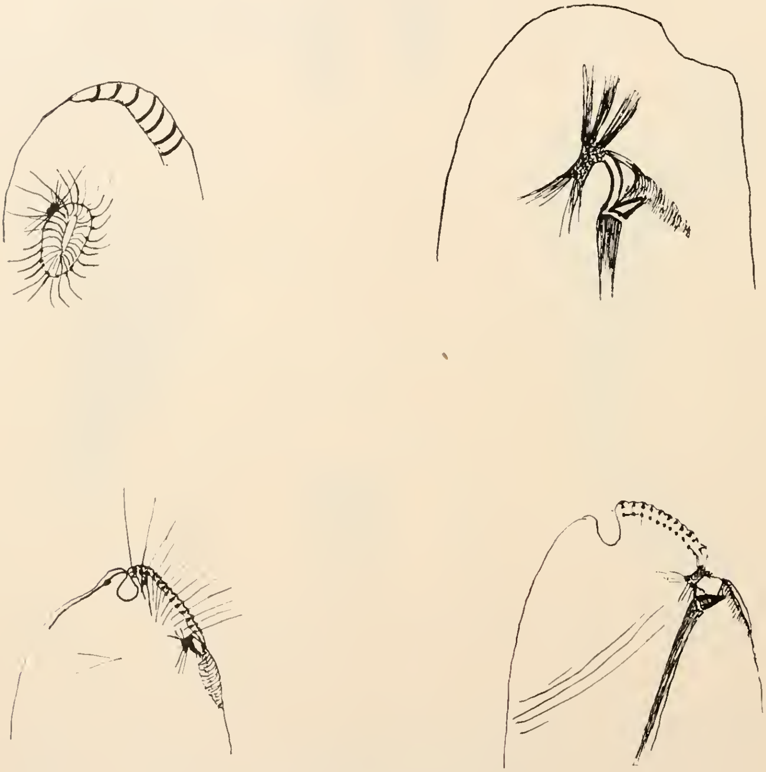
Fig. 7. Circular myoneme surrounding the month, with traversing fibers.
Fig. 8. Tangential section through mouth region, showing relation of basket to myoneme and fibers.
Fig. 9. Fibers in caps of trichites and top of trichites.
Fig. 10. Caps removed from the trichites of the basket. Note fan of fibers.

The macronucleus is divided into two parts, the anterior part taking the stain more lightly than the posterior half. In the resting stage, the granules in the anterior half form a complete horseshoe. These granules change in size and position as cell division proceeds. There are, in addition, many small granules. The posterior half has a large division center, and many other smaller granules. Between the two halves there is a split, or querspalt. Often a granule is found in this split, and it has been observed to divide. The two granules migrate into the cytoplasm. It has not been possible to follow their further history. In division, the split disappears, the division center moves up the center of the nucleus, pulls out and divides. All of the stages of division have not yet been worked out.



FIGS. 11, 12, 13, 14. The Motorium, and its relation to other structures.

The position of the micronucleus is uncertain. Entz pictures it on the side of the nucleus in *Chlamydodon cyclops,* and Erlanger shows it imbedded in the anterior end of the macronucleus. It is quite small, and some of my preparations show it at the posterior end of the macronucleus, and some at the side. It cannot be seen in all preparations.

THE NEUROMOTOR APPARATUS

The first part of the neuromotor apparatus to be observed was a system of fibers around the mouth or oral opening, Figs. 2, 6, 7. As described above, the pharyngeal basket in *Chlamydodon* is very large and heavy, and the circular myoneme around the mouth is seen to be traversed by many fine fibers, with a small granule for each one on the edge of the myoneme, Fig. 7. In only one or two favorable preparations could it be seen that these fibers run out into the cytoplasm in the general direction of the "railroad track," Fig. 11. With the aid of micro dissection needles, it is possible to lift off the ventral surface of the animal. If this is

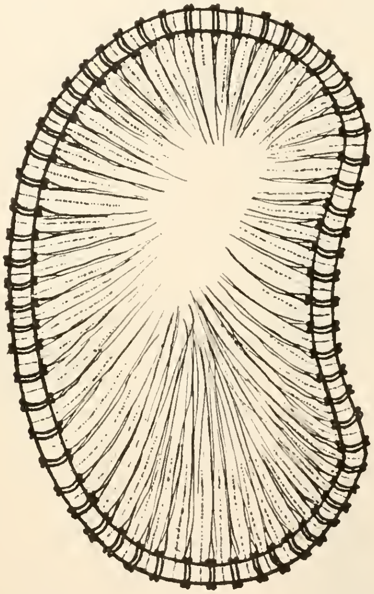


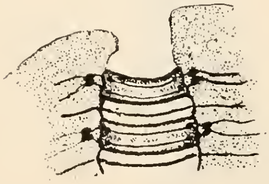
FIG. 15. Diagrammatic representation of the relation of fibers to the "railroad track."

then properly stained, it is seen that the myoneme around the mouth is continuous with the body wall, but much thicker. The fibers from the myoneme extend under the rows of cilia. They are very fine, and it is impossible to trace them far.

Fibers run from one trichite to the next, and there are cross fibers on each of the caps, Fig. 9. These fibers all come together in two heavy strands, joining other fibers around the mouth, and connecting the motorium at the lower right-hand corner, Figs. 12 and 13.

The basket seems to be lined with a very thin membrane, and when the caps are removed from the trichites, fibers can be seen in this lining, Fig. 10.

The motorium is just below the anterior end of the basket, and is placed a little slantingly, Figs. 11, 12, and 13. It is a bilobed mass, which stains with hæmotoxylin. It, and the other fibers in the system, stain bright red with Mallory's connective tissue stain.



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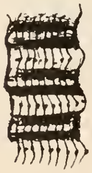

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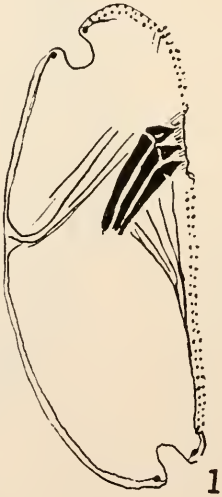

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FIG. 16. Portion of the "railroad track" greatly enlarged.

FIG. 17. Portion of the "railroad track" dissected out and flattened. Shows peripheral fibers.

FIG. 18. Section showing dorsal and ventral fibers from the "railroad track."

It is impossible to see the motorium in the whole mounts. On account of its proximity to the pharyngeal basket, it was overlooked for a long time, for the basket is heavy, and when destaining is carried on long enough to differentiate it, the motorium is destained. In sections, however, the structure is easy to see. With the aid of microdissection needles, the basket can be dragged out whole, and the motorium is sometimes pulled out with it. It is then seen to be a refringent body, while in stained sections its general structure appears granular. Fig. 12 shows the fans of fibers joining the motorium.

The peculiar organelle known sometimes as the "striped band" or "railroad track" mentioned above, has a very complex system of fibers connected with it. Sections show the fibers very well, but their paths are hard to trace on the ventral side on account of the presence of the cilia.

At each end of each trichite, there is a plate or mass, Fig. 16. In sections this mass appears single, Figs. 13 and 18; when viewed from the ventral side of the animal, it appears bilobed or double, Fig. 16. There is a possibility that when the granules were observed from the ventral surface, they were in the process of division, but, when they were seen at all from this angle, they appeared double. The presence of fine rows of cilia on the ventral surface obscures everything; on the dorsal surface the body is much curved in the region of the railroad track, making it impossible to see the granules or masses so close to the organelle. The fibers that go through the trichites are connected with these masses. Fig. 15, in a somewhat diagramatic way, shows the relation of one set of ventral fibers with the masses and the trichites. There are two sets of fibers, one set that enters the trichites, and one set runs through the thinner portion of the "railroad track," Fig. 15. These fibers are from two levels. When the mouth is open, during the ingestion of food, the railroad track changes its shape from a half circle to a closed circle, Fig. 4. From the evidence of the sections, and the observations upon the living material, it is reasonable to suppose that one set of these fibers is connected with the myoneme around the mouth. The presence of ciliary lines and fibers make it almost impossible to trace these fine lines.

The dorsal fibers extend only a short distance underneath the pellicle, then they turn and go in the direction of the motorium.

Only two preparations showed with any clearness the fans of fibers joining the motorium. The basal bodies of the cilia are connected both by longitudinal and cross fibers, Fig. 2 *B*. The longitudinal fibers of the cilia turn in at the zone of origin of the cilia, and connect with the motorium at the anterior end, Fig. 12. This end of the motorium also receives the fibers from the ventral surface of posterior end of the animal. Fig. 15, and some fibers from the mouth region. Figs. 13 and 14. The dorsal fibers join the motorium at the posterior end.

As described above, between each two trichites, there is a thinner portion, and in the center of this is a series of small granules. The granules are connected with fibers which pass over the trichites to the next set of granules, Fig. 17. In some sections, the accordion like arrangement is more pronounced than others, suggesting that there is power of movement, a sort of folding of the trichites. No observations on the living material settled this point, but further evidence of the possibility of the movement suggested lies in the fact that the trichites are sometimes closer together than at other times.

Microdissection.

After the location of the motorium, twenty-five animals were successfully cut, freehand, with the aid of micro-dissection needles given to me by Dr. Robt. W. Chambers of Cornell Medical College. Later, Miss Howland, of New York University, operated upon several with the Chambers micro-dissection apparatus.

The cilia of *Chlamydodon* are fine, the anterior ones being longer and easier to observe than the posterior ones. The motorium cannot be seen in unstained specimens but its position with relation to the basket is known, so that it is quite simple to destroy it. If the motorium be destroyed, there is a marked disturbance in the action of the cilia, in no way comparable to the disturbance of the cilia if other parts of the body are injured. The cilia still have their wave like motion, however, and this is to be expected when one takes into consideration the relation of connecting fibers of the cilia, Fig. 2 *A* and *B*. However, the cilia do not reverse after the destruction of the motorium as is usual in intact animals. Isolated pieces behave the same way, as has been observed by Jennings and Jamieson (1902), and by Rees (1922). Animals without conspicuous motile organs are not favorable material for the study of the coördination by means of microdissection.

Summary.

A description of *Chlamydodon*, probably a variation of the species mnemosyne, found in brackish water at Woods Hole, is presented.

There is a complex neuromotor apparatus, including a coördinating center, and systems of fibers connected with cilia, the mouth opening, the phryngeal basket, and the "railroad track."

The connection of the fibers with the organelles and the central mass, or motorium, the behavior of the animal after the destruction of the motorium, seem to suggest a coördinating function for the system.

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OBSERVATIONS ON THE FOOD REACTIONS OF *ACTINOPHRYS* SOL.

JAMES BURDINE LOOPER.[1]

INTRODUCTION.

Observations on the ability of the protozoa to choose their food date back to 1838 at least. At that time Ehrenberg observed that various organisms ingest carmine which, as food, is nothing more than inert matter. From 1838 until 1902 most of the workers in this field held that protozoa do not possess the power of choosing their food. Even at the present time, as stated by Visscher ('23) in the introductory to his work on *Dileptus gigas,* it is evident that the selection of food has thus far been positively demonstrated in a very few forms.

Kepner and Whitlock ('21), in their work on *Amœba,* give proof of qualitative efforts on the part of *Amœba* to meet certain contingencies. Up to the present time proof of such qualitative effort in other rhizopods has not been recorded so far as I have been able to determine.

This work was done under the direction of Professor William A. Kepner, to whom I am deeply indebted for helpful suggestions regarding these experiments and the preparation of this paper. I am also indebted to Professor Bruce D. Reynolds for valuable criticisms and suggestions.

CULTURE METHOD.

The best cultures were obtained in an infusion made by boiling three grains of wheat for five minutes in 100 cc. of filtered spring water. The infusion, along with the three grains of boiled wheat, was placed in a small, clean, glass aquarium and allowed to stand at least twenty-four hours before the animal was introduced. After about a month had passed *Actinophrys* could be secured from such a culture usually in abundance. The cultures were kept covered with thin sheets of plate glass and were kept in the darkest portion of a well lighted room. To keep the cultures going it was found necessary to add to each culture 5 to 10 cc. of boiled spring water per week. The Heliozoa subsisted chiefly upon *Peranema* and *Colpidia*, the progenitors of which were introduced when the cultures were inoculated.

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Methods of Observations.

All observations were made under the compound microscope —the 4 mm. objective and number 10 eyepiece being used in practically every observation made. A number 10 eyepiece containing a corrected micrometer, and having a camera lucida attached was kept at hand in case sketches or measurements were to be made.

Cultures in Petri dishes were examined under the 16 mm. objective. In this way observations were obtained on movement and general behavior in a habitat to which the animals were thoroughly accustomed.

A few observations were made on specimens in a drop of water on an ordinary micro-slide under a cover glass, but most observations were made on specimens mounted in a hanging drop. The former method is inconvenient because water has to be supplied to compensate for evaporation. This often disturbed the *Actinophrys* in such a way as to cause it to lie quietly for some time, hence, much valuable time may be lost. The hanging drop method alleviated this trouble once the mount was properly made. To prepare the mounts, first a glass ring about 1 cm. in height and 1½ cm. in diameter, was placed on a clean slide and attached thereon by applying vaseline around the proximal end of the ring. The ring was then supplied with well-oxygenated water until the meniscus came up to half the height of the ring. Clear water in which *Elodea* or *Chara* was actively growing was found to be best. The supply of oxygen in the water in the ring tends to replenish the supply in the hanging drop as it is used up by the mounted *Actinophrys sols*. The desired number of the above mentioned specimens was then isolated by means of a capillary pipette and placed on a clean number one coverglass. Next, with another capillary pipette, the desired objects of prey were applied to the drop on the coverglass containing *Actinophrys sol.* The upper end of the ring, which had previously been prepared, was smeared with a small amount of vaseline. The coverglass was then inverted and gently pressed down on the ring. Mounts, prepared as described above, often lasted two weeks for observation. In observing these mounts under the 4 mm. objective, better lighting was obtained by removing the sub-stage condenser and employing the concave mirror.

Special Pseudopods.

The special pseudopods for taking in prey are always composed of hyaline ectoplasm. There are three general types of these special pseudopods. First, if the object to be ingested is very small and relatively motionless, a small, straight pseudopod is extended, and upon coming in contact with the object spreads out into a cup which encircles and closes in closely on the object. These pseudopods often resemble a dipper if the protruding end happens to pass beyond the food object before contact is made. Second, if the motionless object be large, a wide, hyaline outgrowth (Fig. 1, 1 *a*) of protoplasm advances towards the prey. When it gets quite near to, or comes in contact with the prey, the tip of this special pseudopod expands in all directions laterally. These lateral expansions close about the large motionless food object usually in close contact with it. In the few instances when close contact may not be made about the large motionless food object, the food vacuole is always decidedly smaller than is the case with animate objects mentioned in the next or third class. Third, large sack-like pseudopods (Fig. 2, 1 *a*) are sent out to encircle active animals. The victim is often cut off from a means of escape by a palisade of rays on one side, and by the approaching special pseudopod on the other.

Reactions to Inanimate Objects.

In experiments on reaction to inanimate objects, only such materials were used that were thought to be non-toxic to *Actinophrys*. In addition, the materials chosen were of such nature as to be easily distinguishable in the vacuoles. The following were tried:

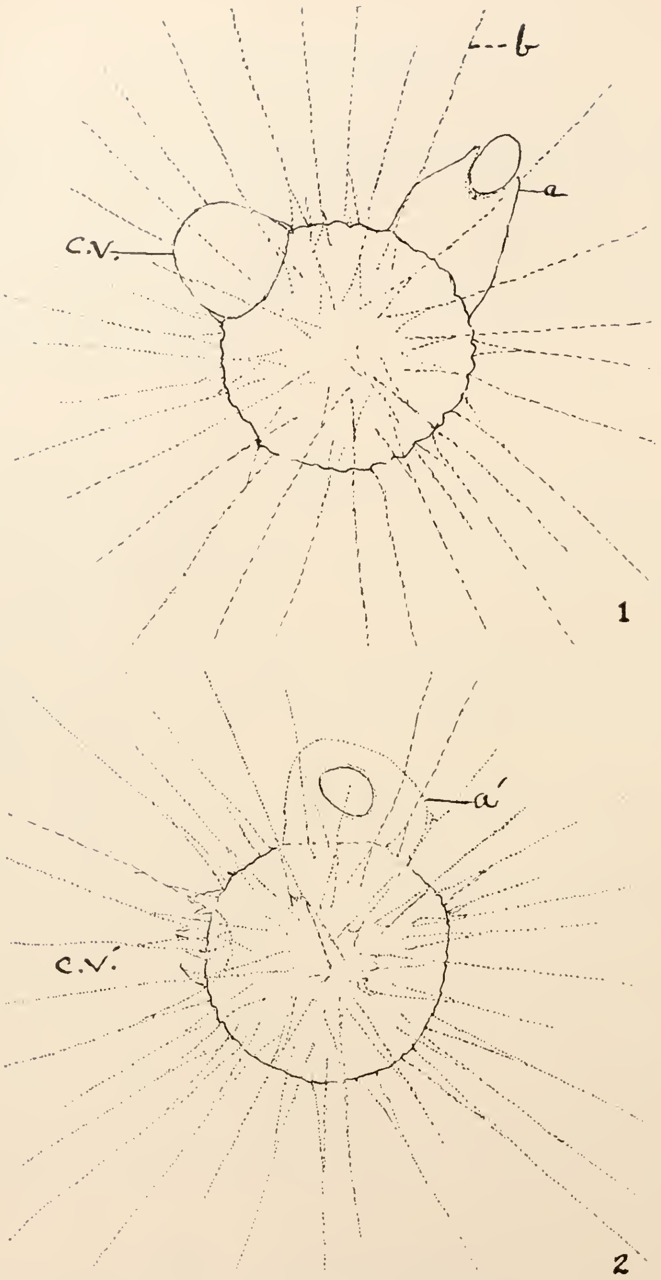

Fig. 1. Sketches illustrating the ingestion of an inanimate object—granule of wheat starch.

1. *Actinophrys sol* with the granule partially enclosed by the food-getting pseudopod, *a*; *b*, ray; *c.v.*, contractile vacuole.

2. Same specimen one half hour later in which *a* has grown to *a′*. *c.v.′*, Protoplasmic projections representing broken wall of emptied contractile vacuole. (× 825.)

carmine, alizarine, powdered glass, plain wheat flour, corn starch. and powdered graphite. Ten experiments were tried with each of these materials, but evidence of ingestion was seen only in the experiments where carmine, wheat flour, and corn starch were used. In the experiments with each material care was taken to try individuals which were in different physiological conditions; *e.g.*, small, underfed specimens, well fed specimens, and specimens which were accustomed to different types of food. Only experiments with materials which *Actinophrys* ingested will be recorded in this paper. The following tables contain the results:

TABLE I.

REACTIONS TO CARMINE.

Experiment.	No. of Individuals in Hanging Drop.	No. which Did Not Take Any Particles within 2 Hrs.	No. Taking Only One Particle within 2 Hrs.	No. Taking Two Particles within 2 Hrs.	No. Taking Three Particles within 2 Hrs.	No. Taking More than Three Particles within 2 Hrs.
1	8	5	2	1	0	0
2	5	4	1	0	0	0
3	11	9	1	1	0	0
4	7	5	1	0	1	0
5	4	3	1	0	0	0
6	5	3	2	0	0	0
7	6	5	0	1	0	0
8	3	2	1	0	0	0
9	15	12	2	1	0	0
10	5	4	1	0	0	0
Total	69	52	12	4	1	0
Per cent	—	75.4	17.4	5.8	1.4	0

In each experiment observations were made as soon as the hanging drop mount was made, then every fifteen minutes thereafter for a period of two hours. In no case did it seem probable that ingestion followed by egestion took place within the time between observations. This is because *Actinophrys* reacts slowly.

Table I. shows that fifty-two individuals (75.4 per cent.) did not take carmine at all. Twelve individuals (17.4 per cent.) took only one particle of carmine. The number taking two particles was four, only one individual took as many as three particles, while none took more than three. Hence, it is evident that carmine was ingested by only about one fifth of the total number of individuals, and these took carmine in relatively small quantities.

I was fortunate in seeing eight of the specimens ingest the particles. In each case small, straight, food-getting pseudopods were extended and the particles were closely embraced. This phenomenon will be referred to again later.

TABLE II.

REACTIONS TO WHEAT FLOUR.

Experiment.	No. of Individuals in Hanging Drop.	No. which Did Not Take Any Particles within 2 Hrs.	No. Taking Only One Particle within 2 Hrs.	No. Taking Two Particles within 2 Hrs.	No. Taking Three Particles within 2 Hrs.	No. Taking More than Three Particles within 2 Hrs.
1.........	5	2	1	2	0	0
2.........	5	3	2	0	0	0
3.........	7	4	2	0	1	0
4.........	4	2	1	0	0	1
5.........	9	5	3	1	0	1
6.........	7	6	0	1	0	0
7.........	10	7	2	0	1	0
8.........	3	2	1	0	0	0
9.........	6	3	2	1	0	0
10.........	8	5	2	1	0	0
Total..........	64	39	16	6	2	2
Per cent........	—	61	25	9.3	2.3	2.3

In Table II. are found the results of ten experiments with plain wheat flour. Thirty-nine individuals (61 per cent.) did not ingest particles of the flour. Twenty-five per cent. ingested only one granule each, and three tenths per cent. ingested two granules, while two and three tenths per cent. ingested three and four granules, respectively. None were observed to take in more than four granules.

The experiments with flour were carried out under practically the same conditions as the experiments with carmine. However, the results of the former tend to show that particles of wheat flour are taken in by a larger number of individuals, and in relatively larger amounts, than carmine was by individuals under similar conditions. This can probably be explained by the fact that particles of wheat flour may be coated with a thin film of protein material which *Actinophrys* is able to digest and assimilate, though I was not able to detect any change in granules which lay in the food vacuoles for as long as five days.

The type of pseudopod employed in engulfing particles of wheat flour belongs to the second class previously described for the taking in of relatively large motionless objects. As with the carmine particles, the pseudopod closely embraces the particles of flour, though the process is very much slower. The slowness of ingestion is probably due to the larger size of the particles.

Table III. contains the results of ten experiments with corn starch. Fifty-eight individuals (85.3 per cent.) did not take in granules at all. Eleven and seven tenths per cent. engulfed only one particle each. Three per cent. took in two particles each, while none took in more than that number. By referring to Tables I., II., and III., it may be easily seen that these results show that corn starch was taken in by fewer individuals than either carmine or wheat flour.

Table III.

Reactions to Corn Starch.

Experiment.	No. of Individuals in Hanging Drop.	No. which Did Not Take Any Particles within 2 Hrs.	No. Taking Only One Particle within 2 Hrs.	No. Taking Two Particles within 2 Hrs.	No. Taking Three Particles within 2 Hrs.	No. Taking More than Three Particles within 2 Hrs.
1	12	10	2	0	0	0
2	8	8	0	0	0	0
3	5	4	0	1	0	0
4	9	7	1	1	0	0
5	9	8	1	0	0	0
6	3	3	0	0	0	0
7	6	5	1	0	0	0
8	5	3	2	0	0	0
9	5	4	1	0	0	0
10	6	6	0	0	0	0
Total	68	58	8	2	0	0
Per cent.	—	85.3	11.7	3	0	0

The method of ingesting corn starch was observed to be practically identical with the method referred to under experiments with wheat flour, viz., inanimate particles are closely embraced by the food-getting pseudopods.

Since it is more convenient to merely state the results of the following experiments, tables will not be given. In all experiments the procedure as outlined under the reactions to inanimate objects was followed and the data were obtained in a corresponding manner.

Reactions to Motionless Animate Objects.

Under this heading reactions to living yeast, motionless algæ, and *Euglena* cysts are recorded.

A. Desmids.

a. Scenedesmus.—In experiments with *Scenedesmus* forty-eight *Actinophrys* were involved. Sixty per cent. did not take the algæ. Those engulfing only one mass constitute thirty and three tenths per cent. The remaining nine and six tenths per cent. of the individuals engulfed two masses each. These desmids were taken in by the same type of pseudopod as were the inanimate objects.

b. A Larger; Desmid.—Fifty animals involved in ten experiments were tried with this desmid. Only four per cent. attempted to engulf the larger algæ, and in one case only was the attempt successfull. In this case one *Actinophrys* sent out a pseudopod and partially surrounded the plant but was not large enough to completely surround it. This animal, while attempting to surround the desmid, was joined by another animal. This union appeared to be a complete fusion of the cytoplasm of one with that of the other, the nuclei remaining separate. After this fusion the pseudopod of the first animal was enlarged to completely surround the desmid. The desmid remained in the food vacuole of the multiple individual for a period of five hours and was then slowly forced out through the outer membrane. Nothing remained of the plant except the cellulose wall and a few particles which remained on the inside of it. A similar phenomenon in the Rhizopods was referred to by Delage and Herouard ('96), as a "*Societe de Consumation.*" Distaso ('08) called attention to the grouping of *A sol* for the capture of large food objects.

B. Yeast.

In experiments with yeast seventy-two animals were used. Twenty per cent. of these took in one or more of the plants.

Since yeast plants are so small it was impossible to keep check on the number taken in. When taking in objects of the above kind the pseudopod was of the small straight type referred to previously in this paper. The plants in all cases observed were closely embraced by the pseudopod until digestive fluid began to collect around them, filling a space between the outside of the plants and the wall of the vacuole.

C. *Cysts of Algæ.*

Euglena cysts and cysts of *Chlamydomonas* were taken in in practically the same proportion. In each case one cyst each was taken in by fifteen individuals (20 per cent.). Four per cent. took in two cysts each, while only one per cent. ingested three each. There were not any taking in more than three cysts. The close embrace was employed in all ingestions observed.

The experiments with motionless animate objects show that *Scenedesmus* is taken more often than either living yeast, *Euglena* cysts, or cysts of *Chlamydomonas;* also, that a larger desmid was rarely chosen as an object of food. The ingestion of the larger algæ required the coöperation of two or more individuals.

Reactions to Moving Animate Objects.

In reacting to moving animate objects *Actinophrys* presents an entirely different type of food-getting pseudopod. Previously, this was referred to as a relatively large, cup-like pseudopod. Instead of the close embrace of the object by the pseudopod, which was referred to under reactions to inanimate objects, we have what may be referred to as a "subtle embrace." A description of the capture and ingestion of a *Colpidium* will serve to make clear what is meant by a "subtle embrace."

While watching the movements of an *Actinophrys* a *Colpidium* was observed to encounter the tips of some of the rays of the former. The *Colpidium* became motionless almost instantly, as if completely paralyzed by the encountered rays. While watching to see the ultimate fate of the ciliate a relatively large mass of cytoplasm was observed to be protruding from the surface of the *Actinophrys*. This mass, which proved to be a food-getting pseudopod, came forward as a single mass until it was four micra

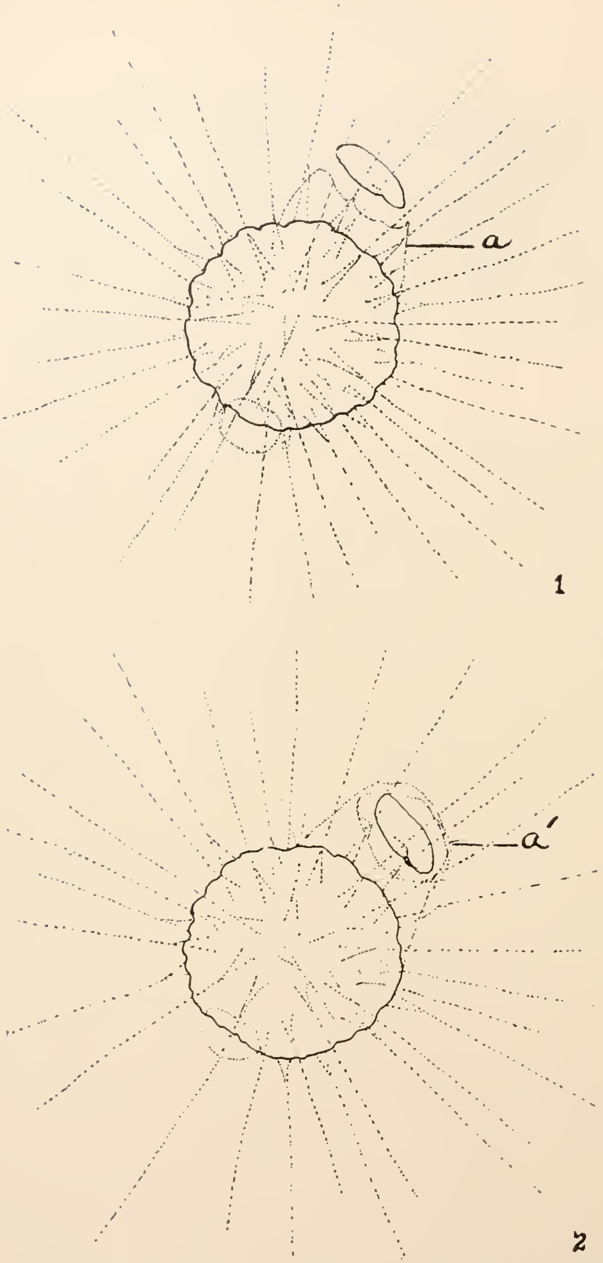

Fig. 2. Sketches illustrating the ingestion of a moving animate object—*Chilomonas.*

1. *Actinophrys sol* with a pseudopod, the contour of which is becoming adapted to that of the prey before coming into contact with the latter. *a*, food-getting pseudopod.

2. Same specimen fifteen minutes later. *a* has grown to become *a*′, which shows wide detour about the motile prey. (× 750.)

away from the quiet *Colpidium;* then, without approaching farther, it gave off two branches (Fig. 2, 1 *a*). These branches continued to protrude, encircling the victim, and fusing on the opposite side of it. Thus far, the *Colpidium* was not approached closer than two micra by the branches of the engulfing pseudopod. Hence, the pseudopods were thrown subtly, but not intimately, around the prey. Immediately after the fusion of the pseudopods beyond the prey they spread in such a way as to form a hollow sphere or vacuole containing the *Colpidium* on the inside (Fig. 2, 2). The vacuole then contracted around the prey, which in the meantime became stimulated to exhibit frantic efforts, apparently for the purpose of escape. These efforts were kept up for a period of at least five minutes before the imprisoned animal finally became quiet again. The vacuole containing the prey was eventually incorporated into the body and grew smaller as digestion proceeded, and disappeared completely when the indigestible particles were finally ejected through the cell membrane of *Actinophrys.*

A. Ciliates.

Colpidium.—Ten experiments with *Colpidia* reveal the fact that forty individuals (50 per cent) took in one or more *Colpidia.* Thirty-seven and seven tenths per cent. engulfed one *Colpidium* each. Twelve per cent. took in two specimens each; while only three tenths per cent. engulfed three each. Apparently, the reason why there was such a small per cent. taking in as high as three each lies in the fact that *Colpidium* is relatively too large to be engulfed in larger numbers by *Actinophrys* within two hours.

Loxocephalus granulosus.—*Loxocephalus* is taken in very readily and in relatively large numbers by *Actinophrys.* In the experiments with the above mentioned ciliate only thirty-six (40 per cent) of the Heliozoans failed to ingest one or more. Thirty per cent. of the individuals engulfed one ciliate each. Two each were taken in by twenty per cent., while ten per cent. were observed to have three vacuoles containing one *Loxocephalus* each. The type of pseudopod used in taking in this organism was observed to be practically identical with the type described under reactions to *Colpidium.*

Stylonychia mytilus.—This ciliate is much larger and stronger than *Actinophrys.* Some of the cilia have been modified to form bristle-like cirri which may be used as organs of offence, or defence. Hence, it is hardly possible for *Actinophrys* to capture such an organism single-handed, and so far as known no one has ever observed such. I have seen four different cases where individual *Stylonychia* were engulfed by a group of *Actinophrys.* These groups in two cases were assembled after one *Actinophrys sol* had tried to capture and ingest the larger and stronger animal. In the other two cases the groups had been previously formed. In no case was a *Stylonychia* taken in by a group of less than four individuals. The group action here, if it may be called group action, differs in at least one respect from the association of individuals described by Kepner ('25). In the case he reported, three individuals united to take in a motionless desmid, and the pseudopods of the former embraced the latter closely. In the case of a group taking in a *Stylonychia* the pseudopods presented the phenomenon of the loose, or subtle embrace described under reactions to *Colpidium.*

Other Ciliates.—Ten experiments were carried out with *Frontonia lucas, Nassula ornata,* and *Spirostomum ambiguum* respectively. Some of the experiments in each case were kept under observation for five days. However, in no case were any of the above-mentioned ciliates engulfed by *Actinophrys;* while *Colpidia* and *Chilomonads,* which happened to be among these ciliates, were all engulfed sooner or later. This certainly indicated a definite discrimination in regard to the type of ciliate taken in. On what is this discrimination based? Whatever the basis, it is hard to believe that either *Frontonia, Nassula* or *Spyrostomum* would be more difficult, from a mere physical standpoint, to capture and engulf than *Stylonychia.*

B. Reactions to Flagellates.

Chilomonas paramecium.—Reactions to this flagellate were practically the same as described under reaction to the ciliate, *Colpidium.* Forty-one individuals (51 per cent.) of *Actinophrys sol* did not engulf *Chilomonas.* Of the remaining forty-nine per cent. forty per cent. engulfed one each. Six per cent. took in four each.

Peranema trichophorum.—*Peranema* proved to be engulfed very readily by *Actinophrys.* Only twenty-five individuals (42 per cent.) failed to take in this flagellate. Thirty-five per cent. engulfed one per individual. Nineteen and one tenth per cent. took in two each, and three per cent. three each. In no case were as many as four *Peranema* engulfed by an individual. The complete process of capture and ingestion was observed in eleven cases. In each the subtle embrace was employed.

Euglena viridis.—*Euglena viridis* was taken more readily than any other food material. Thirty-five individuals (58.4 per cent.) of the Heliozoan ingested one each. Ten per cent. took in two each, and one per cent. three each.

Euglena, as a rule, was embraced more closely than either of the other moving animate objects. I have not been able to observe definitely why this was the case. However, this deviation from the usual procedure in ingestion of moving animate objects probably has some connection with the fact that *Euglena* was often brought in contact through a reaction with the body proper of *Actinophrys* before a food-getting pseudopod was sent out. This contact with the body proper was brought about by a sudden jump of *Euglena* in the direction of the body proper of *Actinophrys* while reacting to a contact with the rays. The exact reason for this sudden jump on the part of *Euglena* has not been determined, though it has been suggested that the long flagellum of *Euglena* became attached to the body of *Actinophrys,* probably, by a mucilaginous property of the latter. Then in an effort to free itself, the *Euglena* pulled itself into contact with the body of *Actinophrys* to which the end of its flagella was fixed. Thus the body of the flagellate became fastened by the same property. If this be true, the food-getting pseudopod which arises from the outer region of the Heliozoan's body, would quite probably adhere closely to the flagellate's body as it encircled it. Should, however, the *Euglena* begin to squirm, the cup of the pseudopod would open up to form a larger cavity around the victim. Thus in case the *Euglena* began to struggle, what was started as a close embrace was forthwith transformed into a subtle embrace.

D. Reactions to Rotifers.

Only individuals belonging to one or two of the small species of Rotifers were taken in by *Actinophrys.* Rotifers are the only multicellular animals which were observed to be captured and ingested by the Heliozoan. Here, as with *Stylonichia,* a group of less than four was not strong enough to capture a Rotifer. I was fortunate to see the complete struggle involved in the capture and ingestion of five specimens by five different groups of *Actinophrys.* Here, as in the case of the capture and taking in of *Stylonychia,* some of the groups were formed after a single individual had become attached to the prey (Fig. 3). In the formation of two of these groups individuals which were entirely out of the low power field of the microscope joined the fray, fusing with their comrades which were already involved. In one of the above mentioned cases four individuals were not in the field when the Rotifer was encountered by an *Actinophrys* which was in the field. In the other case, likewise, three were outside of the field of vision. In these cases the rays of the individuals which came into the field of the microscope to join in the feast were not adjacent with the rays of those already involved. This seemed to indicate that there must be some way of sending out a call for assistance when the object was too large and pugnacious to be conquered and swallowed by one individual. The reason that it cannot be said definitely that this is the case is because the outsiders might be drawn in by currents set up in the medium by the floundering of the attacked prey. The fact that a number of *Actinophrys sol* fused to form an aggregate when brought together by agitating the surrounding medium with a glass filament, strengthens the above mentioned idea. Whatever may be the cause of the formation of temporary colonies, the phenomenon is, at least, of interest. By virtue of this fact, it becomes easier to conceive of how multicellular animals may have arisen by the association and coöperation of individual cells. Be this as it may, the formation of multiple individuals by *Actinophrys sol* makes possible the capture and ingestion of larger objects of prey. This is essentially in agreement with Distaso's ('08) observations on *A. sol.* Apparently this temporary colony formation also serves a protective rôle. It is probably of interest to note that an animal so low in the scale of organic life exhibits the phenomenon of coöperation in the fundamental activities of its life.

By comparing the results of the experiments carried out with

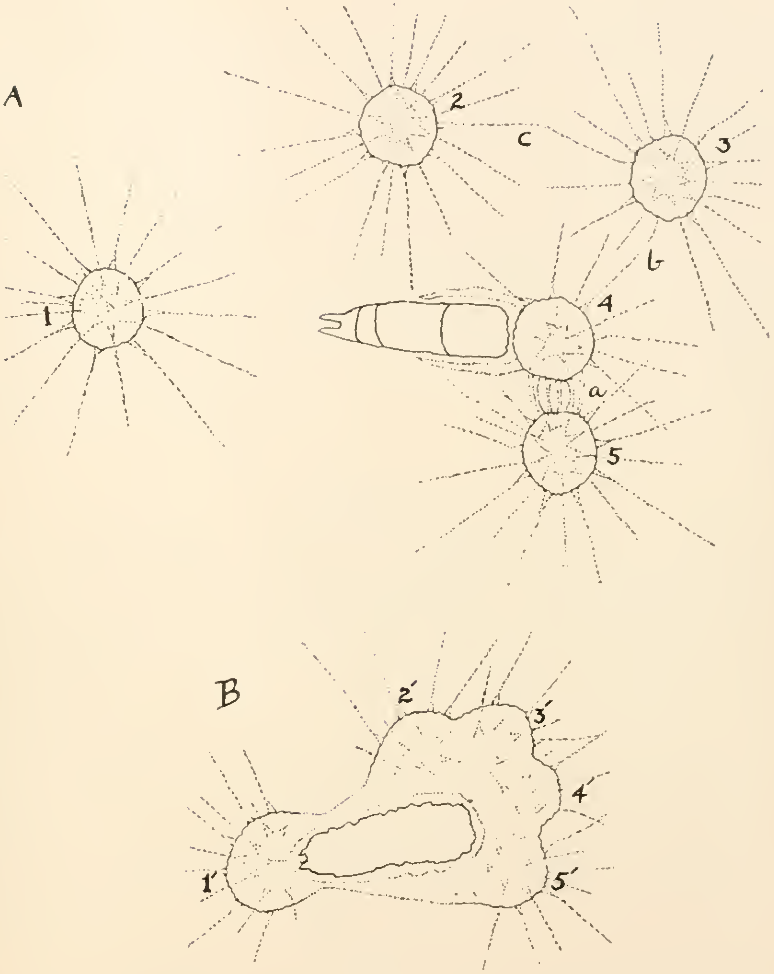

Fig. 3. *A*. Five *Actinophrys sols* later to be involved in the capture and ingestion of a rotifer. Specimens 4 and 5 already show advanced coöperation, *a*. Specimens 3 and 4 show the inception of coöperation through the fusion of two of their slender rays, *b*. Specimens 2 and 3 are also involved in the coöperation through the fusion of two rays at *c*. Specimen 1 is yet independent.

B. Same group showing progress made within two hours. Complete ingestion has been accomplished and some digestion has ensued. 1′, 2′, 3′, 4′, and 5′ shows the position taken by 1, 2, 3, 4, and 5, respectively, of Fig. 3 *A*. (× 260.)

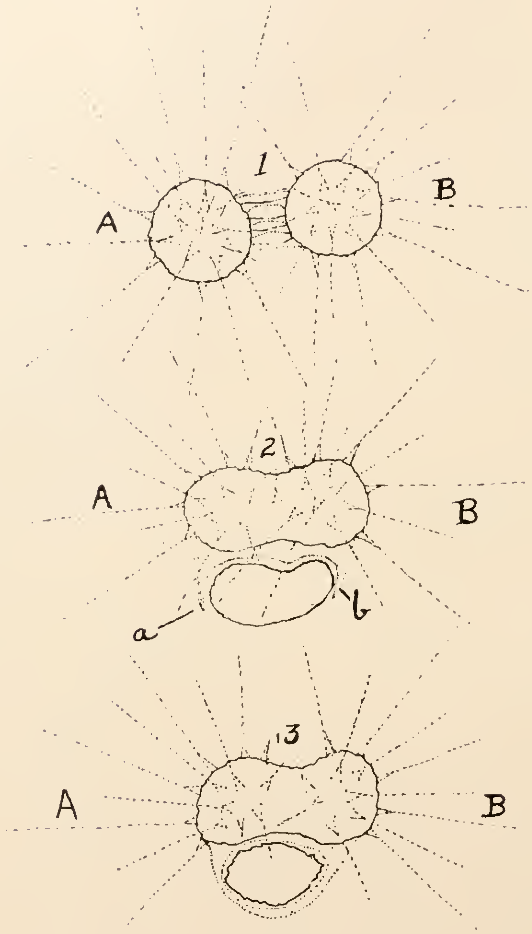

FIG. 4. Sketches illustrating the fusion, also, the coöperation of two *Actinophrys sols* in the capture and ingestion of a *Colpidium.*

1. Pseudopodial fusion in the union of the two individuals.

2. Coöperation of the same two individuals in ingestion of *Colpidium* by one individual, *A*, sending out *a* and individual *B* sending out *b* to form in common one large food-engulfing pseudopod.

3. Same specimens one and one half hour later. Prey engulfed by fusion of pseudopods *a* and *b* about it. (× 325.)

the various types of objects it may easily be seen that *Actinophrys sol* feeds chiefly on the smaller ciliates and on the flagellates. The experiments with moving animate objects show that certain of these objects are eaten more readily and in greater numbers than are others. Difference in the size and vigor of the different food objects, no doubt, plays an important rôle in causing this discrimination. However, it is not probable that difference in size and vigor alone is the only factor causing the discrimination noted. The experiments with moving animate objects also show that such objects are not embraced intimately by the food-getting pseudopods of *Actinophrys sol;* but on the contrary, the moving animate objects, as a rule, are loosely or subtly embraced by the food-getting pseudopods.

SUMMARY.

1. *Actinophrys sol* is essentially omnivorous.

2. Certain inanimate objects are ingested more often than others. Some inanimate objects apparently are not ingested at all.

3. Motionless animate objects are accepted, but not in appreciably greater numbers than inanimate objects.

4. Many free-swimming ciliates and flagellates are readily eaten, but certain ciliates apparently are not taken.

5. A degree of selection or discrimination was exercised between different food objects in every class of materials used.

6. Large objects of prey may be taken in by temporary colonies of *Actinophrys sol* which are formed for the purpose of taking in such objects.

7. As a rule, motionless animate objects and inanimate objects are closely embraced by the ingesting pseudopod; objects which struggle while being ingested are subtly embraced.

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THE HONEY-GATHERING HABITS OF *POLISTES* WASPS.

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Wheeler, in his valuable paper on "Vestigial Instincts in Insects and Other Animals," * gives a résumé of what is known as the honey-gathering propensities of wasps, principally those of the tropics. He also records his own discovery on November 3 of a nest of *Polistes metrica* in New York City, hanging from the eaves of a boathouse. This nest of a few dozen cells had four females, inactive from the cold, clinging to the comb, and six cells contained half-grown, much contracted but still living pupæ. There were small drops of colorless liquid in many of the other cells, and one of them was half full of this substance, which tasted like and proved to be honey of an agreeable flavor. These drops, he tells us, hung suspended in the angles of the cells, but were without any definite arrangement, and varied much in size. "This honey must have been collected some weeks previously from the autumn flowers and stored, now that nectar and insect food were no longer to be had, for the purpose of bringing the few remaining larvæ to maturity. The belated brood undoubtedly accounted for the presence of the female insects at so late a date."

Wheeler assembles the details of the observations on honey-storing by tropical wasps made by Lepeletier, Rouget, Marchal and others, and then goes on to show that in the northern species this habit of honey-storing is a vestigial instinct, and is called into play only when the insect is facing unusual conditions. To use his exact words: "The great quantities of honey collected by the tropical wasps are, of course, stores of provisions for the winter, for, as the von Iherings have shown, many of the species, unlike the northern *Polistes,* do not abandon their nests on the approach of the unfavorable season and start new ones in the spring, but continue to add to their combs and keep on raising their brood throughout the year. The naturalists are unquestionably right in deriving the conditions seen in our northern *Polistes* from those of the tropical species. There can be no doubt that *Polistes* has extended its range into North America and Europe since the close of the glacial epoch. The storing of honey for the winter has been discontinued, and the life of the species has been saved by a new set of adaptations involving the abandonment of the nest, the temporary suspension of the breeding instincts, and the hibernation of a small number of fertilized females. The drops of honey occasionally stored in the nests are all that remains to point to a once very important means of tiding over the flowerless season and preserving the life of the individual colony. Rouget, Brongniart and myself have observed this vestigial instinct only in the autumn, and this would seem to be the most likely time for the feeble display of the old habit.

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* *Am. Journ. Psychol.,* 19: 1–13. 1908.

"Not only has the honey-storing instinct of our northern *Polistes* been reduced to a feeble and useless vestige by the adaptation of this insect to life in a temperate zone, but the nest-building instincts, when compared with those of the allied tropical wasps, show unmistakable signs of similar degeneration."

This interpretation was to me highly fascinating, for I too had occasionally seen similar drops of this clear, honey-like substance attached to the walls of the cells of *Polistes pallipes*. Other things crowded out further investigation of this point until early in 1920, when the above-mentioned paper fell into my hands, and I decided to look fully into the matter, with the following results.

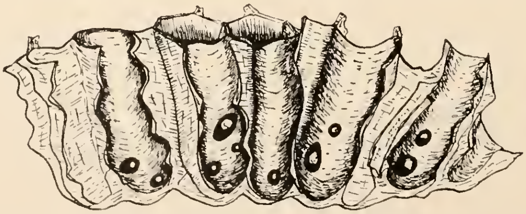

FIG. 1. A cross section of a nest of *Polistes pallipes*, showing globules of honey in the cells. (Exact size.)

Investigation No. 1.—On June 16, 1920, twelve nests of *Polistes pallipes* were found in the old buildings at Wickes, Mo., and one nest was far in among the branches of a closely foliated cedar tree. None of these cells had workers, but each nest was presided over by the queen. A close examination was made of each nest, and the twelve in the buildings showed no evidence of having drops of this jelloid substance in their cells. The one nest out in the cedar tree, however, gave positive evidence. This was a nest of fourteen cells, with the four central ones filled with half-grown larvæ; these cells contained none of the jelloid material, but the ten surrounding cells each contained an egg and from one to three drops of this material glued to the angle of the cell. Some of the globules were quite large. An idea of the relative size when the terms large and small are used may be gained from Fig. 1, which illustrates the globules, exact size. The substance had a decidedly sweetish taste. This was an absolutely newly made nest; there was consequently no possibility that this material could have been left over from the preceding year in an old nest. The twelve nests which gave negative results also contained eggs, so of the thirteen mothers which worked in this neighborhood, only one provided this material for her young.

Investigation No. 2.—The one nest containing the globules in the previous study was taken; the other twelve were undisturbed, with the idea of studying them later. By mid-summer (July 21), the workers had matured and covered the nests, so that observations from day to day were impossible on account of their inaccessibility, the ferocity of the occupants and the poor light. With so important a problem, I could do nothing but knock the nests down with a stick, and after the infuriated wasps had flown back to the place where the nest had been, harvest my booty.

In this study, thirteen nests were taken and carefully examined; ten of these were nests of *Polistes pallipes*, one was *P. variatus* and two were *P. annularis*. Every nest except one of this lot gave evidence more or less abundant to show that all of these three species store drops of this "honey" in their cells containing eggs or very tiny larvæ. Throughout this study I have never found these drops in cells containing larvæ more than a few days old. The data herewith give the details for the thirteen nests, all taken on July 21, 1920.

In the foregoing table, the first ten nests were *P. pallipes*, "*k*" was *P. variatus*, and "l" and "m" were *P. annularis*. These few nests show that all three species follow this habit of supplying

Nest.	Cells Only Begun.	Cells Cont. Eggs.*	Cells Cont. Honey.	Cells Cont. Larvæ.	Cells Cont. Pupæ.	Total Cells in Nest.	Remarks.
a.....	—	12	1	—	—	12	
b.....	—	30	0	—	7	37	
c.....	—	15	14	6	10	31	From 1 to 3 beads of various size. Note: much honey but no larvæ.
d.....	6	30	25	—	—	36	
e.....	—	12	3	5	5	22	
f.....	—	24	24	3	9	36	One to 3 drops of various size.
g.....	—	26	14	5	7	38	
h.....	—	75	49	13	13	101	Drops varied in size, number and consistency.
i.....	—	36	32	6	18	60	
j.....	—	61	49	7	10	78	From 1 to 4 beads, many of them very large.
k.....	—	20	20	10	14	44	From 1 to 3 globules of various size.
l.....	—	22	2	13	4	39	
m....	—	38	1	23	41	102	
13	6	401	234	91	138	636	

* This signifies eggs or newly hatched larvæ less than a day old.

their young with this honey, but of course from such limited data we can have no idea whether any one species is more constant in this habit or furnishes more profuse supplies. The totals show at a glance how common is the occurrence of this food in the egg-cells of wasps even at the middle of the summer season. Out of the 407 cells containing, or about to contain an egg, 234 of them or almost 58 per cent had globules of this honey-like material. The actual occurrence of the honey was probably even more frequent than the numbers indicate, for the reason that the first-day larvæ were included with the eggs, and in some cases they had doubtless already devoured their rations before I counted them.

Investigation No. 3.—In the foregoing experiment, I removed and carried home the thirteen nests of *Polistes,* leaving the adults behind. Three days later, on July 24, I returned to the scene to see if they had rebuilt the nest, and especially to find if, without the stimulus or the aid of the larvae present, they would supply these drops of sweet food in the new cells. The details of their behavior in rebuilding are given elsewhere, but here I record that of the nine colonies which did rebuild (eight of *P. pallipes* and one of *P. variatus*) each nest had from fourteen to twenty-two cells, all very shallow at this stage, but each and every cell contained its globule of jelly along with its egg.

Investigation No. 4.—Records of two nests taken at Wesco, Mo., on August 3, gave the following data. The first was a nest of *P. variatus*, which contained thirty-five cells with eggs, sixteen cells with larvæ and forty-four with pupæ, and had about a dozen adults on the nest. This large nest gave absolutely no evidence of the presence of the honey. The other was a nest of *P. pallipes,* which was in all probability the second one made by this colony, since at this late date it had only small, newly-made cells which would hardly hold a half-grown larva, and there were no cells large enough to have harbored the five adults which were then on the nest. These shallow cells, fifty-two in number, gave no evidence of having a supply of honey, although about one half of them contained eggs.

Investigation No. 5.—Since I had removed all of the accessible nests at Wickes, the next scene of operations was at Meramec Highlands, west of St. Louis and about thirty miles distant from Wickes. These nests were gathered about two weeks later, on August 6, 1920. In this collection, fourteen nests (all *P. pallipes*) were knocked from the walls and eaves with long poles and carried

Nest.	Cells Just Begun.	Cells Cont. Eggs.	Cells Cont. Honey.	Cells Cont. Larvæ.	Cells Cont. Pupæ.	Total Cells.	Remarks.
aa....	10	35	35	—	3	48	All ten new (eggless) cells, and 25 of the 35 cells with eggs contained honey.
bb....	—	23	8	32	14	69	
cc....	—	10	6	25	28	63	
dd....	—	11	6	21	22	54	
ee....	10	20	25	32	45	107	All 10 new (eggless) cells, and 15 of the 20 cells with eggs contained globules.
ff....	6	23	25	18	7	54	Again, all 6 empty cells contained drops of honey.
gg....	—	14	10	38	20	72	
hh....	—	54	35	27	20	101	Some of the globules immensely large.
ii....	—	12	10	14	3	29	
jj....	—	10	10	4	3	17	
kk....	28	11	39	8	21	68	All new empty cells and all cells with eggs supplied. 1 to 4 drops per cell, some very large.
ll	—	57	56	16	25	98	
mm...	—	36	15	36	65	137	
nn...	3	4	3	—	—	7	New nest with no workers.
14....	57	320	283	271	276	924	

home for examination. The data from these will also be more convenient if presented in tabular form.

In this table we find that out of 377 cells containing or ready for an egg, 283, or about 75 per cent. of them, contained drops of this honey-like substance.

The next spring, the following additional experiments were made.

Investigation No. 6.—On this date, April 28, 1921, I found forty-two small nests of *P. pallipes* which had only recently been begun by their queens. In only one cell of all these nests did I find one drop of this honey suspended in the angle of the cell. This one drop is sufficient to show that very early in the nesting season the queens sometimes store this substance. At this season the queens were not so bellicose, so instead of knocking down the nests, at intervals for examination I climbed up to the nests and, with the aid of a flash-light, examined the nests and contents *in situ.*

Investigation No.7. On May 13, twenty-nine nests, with the number of cells varying from ten to eighteen, were minutely examined, and fourteen were found to contain drops of honey. Nest No. 1, with thirteen cells each containing an egg, had a drop in each of two cells. One cannot say that this had been placed there for the young about to hatch, for these two cells were adjacent and were at the margin of the nest, whereas the first eggs to hatch are as a rule in the center of the comb; in this case the eggs in the two center cells were even then much inflated and ready to give forth their life. Nest No. 56 had ten cells all containing eggs, the two central ones large and ready to become larvæ. It was in precisely these cells, that the drops of honey were found. Nest No. 15 comprised fourteen cells having eleven eggs and three larvæ in the center cells. Two other cells adjacent to these had each a drop of honey. The larvæ were several days old, and no evidence remained in their cells to tell whether or not they had had this food in their infancy. The eggs provided with honey were inflated and ready to hatch next. Nest No. 22 had ten cells each containing an egg; the largest of these, already swollen with its embryo, was provided with a drop of honey in its cell.

Thus we see four nests out of twenty-nine at this early date containing honey-drops; I suspect that the larva, upon emerging, gets its first meal from this food, and in three of four of the cases observed on this date this material had been placed in the cells containing first-laid eggs, the larvæ from which would be first in need of food. However, since only about one-eighth of the nests examined contained honey in even a part of their cells, it seems very likely that some of the young lack the advantage of this food, or at least do not have it waiting for them before their birth. The most interesting bit of evidence that this day gave forth, however, was the one cell, which on April 28 had contained a drop of honey, on this date, May 13, contained a small larva but no honey! The period of incubation in this species has elsewhere been found to be a little more than two weeks, so it is not surprising if the mother cannot estimate exactly the date of the need of food for each of her young.

Investigation No. 8.—The accompanying table summarizes the details of the observations on twenty-seven nests on May 27 and 28, 1921. At this time, when the nests were larger and contained more life, I found less of the honey; it occurred in only three of the twenty-seven nests, and as usual only in the cells containing eggs; out of 142 cells only 13 had drops of honey. It seems at first puzzling that a mother would supply this extra provision to some cells containing eggs, and not supply it to all, and that if one mother is so provident, not all are likewise. All were *pallipes.*

I previously formed the hypothesis that all young, when they first hatch from the egg, must be fed this material probably predigested by the mother; that frequently she is on hand at the time and administers the dose personally, and that sometimes she gets an over supply or gathers it before the young are hatched, in which event she relieves herself of it by placing it in the angles of the cells containing the eggs which will soon hatch. The following notes will to a degree bear out the first part of this hypothesis.

While restlessly waiting for a belated train at a rural flag-station at 10 A.M., I fell to watching a queen which had charge of a nest built on the wall of the station shelter. I noted that repeatedly she inserted her head deep into two cells and kept it there far out of sight for periods of three to seven minutes. I knew that trophallaxis occurs between the larvæ and the workers, but this queen had crowded her body so deep into the cells as to completely

Nest.	Cells Just Begun.	Cells Cont. Eggs.	Cells Cont. Honey.	Cells Cont. Larvæ.	Cells Cont. Pupæ.	Total Cells.	Remarks.
51....	—	3	—	13	—	16	
69....	—	11	—	5	—	16	
9A..	—	5	—	6	—	11	
11....	—	2	—	11	—	13	
12....	—	7	7	7	—	14	Honey with all the eggs; none with the larvæ.
1....	—	4	—	10	—	14	
2....	—	10	—	2	—	12	
3....	—	—	—	16	2	18	
13....	—	8	—	10	—	18	
8....	—	2	—	7	—	9	
35....	—	7	—	8	—	15	
34....	—	5	—	10	—	15	
31....	—	6	—	12	—	18	
54....	—	18	6	14	—	32	Honey all in egg cells; this nest had 3 queens.
14....	—	2	—	10	—	12	
15....	—	—	—	10	2	12	
21....	—	4	—	4	—	8	
17...	—	6	—	6	2	14	
58....	—	1	—	10	4	15	
59....	—	2	—	10	1	13	
20....	—	2	—	10	—	12	
23....	—	3	—	11	—	14	
21....	—	—	—	10	—	10	
72....	—	4	3	—	—	4	
73....	—	12	—	—	—	12	
74....	—	12	—	—	—	12	
22....	—	6	—	8	—	14	
27....	—	142	13	220	11	373	

cover the thorax; this indicated at once that the cell must contain only a very small organism, and thus the present process could not be trophallaxis. Presently she removed her head from the cell from time to time and went about examining three other cells which contained eggs; six other cells nearby contained good-sized larvae, but these she ignored. Her attention was always centered about the two cells whose contents I could not see; whenever she left one or the other of these two cells, she would poke her head momentarily into each of the three egg-cells and then go back to the others. When visiting the egg-cells, she would always vibrate the antennæ, bring them close together and point them in front of her head as she entered, insert the head for an instant and withdraw, repeat the process exactly for the other two, and then return and creep deep into one of the mysterious cells for several minutes. When she withdrew from either of these, she moved her mandibles

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in a significant manner, but very different from the "licking-her-chops" manner of the workers after they take the liquid from the larvæ, for in the latter case not only were the mandibles in motion, but also the labium, palpi, etc., were actively registering satisfaction or function.

The approaching train brought the observations to a sudden close. Hastily I pulled out the mother wasp and threw the gleam of the flashlight deep into the cells, and lo! in the far corner of each lay a tiny larva, hardly larger than an egg! I could solve the mystery of her long stops in these two cells only by concluding that she was feeding them with the material from her own mouth, since I was sure she brought nothing to the cells in her jaws. I pondered on the number of times she had poked her head inquiringly into the three cells containing eggs; then I realized that her frequent visits here were probably for the purpose of ascertaining if they had yet hatched or were ready for her attention. The larvæ she did not need to mind just now, but the new-born twins and the eggs had all her attention.

My most recent observations have led me to believe that the queen does not deposit this honey with the intention of making a self-feeder for the larva, so that when the egg hatches the larva has only to thrust its head into it and imbibe, but rather that she stores it here temporarily where it will soon be needed and where she can easily administer it to the waspling as soon as it is ready. The ground for this conclusion is the fact that these drops are almost always placed far below the egg in the cell, or on the opposite wall, so that it is quite impossible for the new-born larvæ to reach them. I have seen dozens of young larvæ in early spring nests which had been brought into the laboratory, die with large drops of honey before their very eyes but out of their reach; since the tail of the larva is glued to the wall, its progression to the drop is impossible. In contrast to this, I have seen a few larvæ survive on this substance when it was artificially placed near their mouths. I have also fed them on that taken from other cells; they ate it readily when served to them on a pin-head. I have also assisted larvæ to get this food by pressing the opposite wall of the cell toward them, bringing the drops nearer to their mouths; and in the past two years I have actually seen two cells in which the drops of honey were placed very near to the larvæ, and they were stretching down and actually imbibing the substance.

The above instances indicate that the newly-hatched larva gets its first meal from the mouth of its mother. This of course can be independent of the storing of the honey as we find it in some cells, but it might point very definitely to the origin of this habit. How logical it seems for all the mothers to feed their new-born young upon predigested food from their own crops, and how possible it could be for the crops to be filled too full or filled too soon. In that event the next logical step would be for the mother to dispose of this surplus by placing it in the cells where she would normally get rid of it at an early date. While one does not wish to speculate too far, one can also see the possibility of another pretty adaptation, in that mothers with only a few offspring at that stage can feed the delicate food to them personally, but when the young are too numerous and the mother's attention must be divided, food may be supplied to them in advance—a habit well known among the solitary wasps. To go further, how possible it is for a social species to show vestiges of a habit of the solitary species,[1] *i.e.*, to supply the egg with food in sufficient amount to carry it to maturity.

Investigation No. 9.—On June 21, 1921, I examined the contents of 20 nests of *P. pallipes*. The egg-laying was over for the time being. It seems that the queen of this species spends her time at this season in caring for the young, and not in adding new cells and depositing more eggs. It was then thought that perhaps the building activities would be resumed after the workers emerged. Only six of the twenty nests contained a very few eggs; hence one would expect proportionately fewer drops of honey in the cells. That is precisely the condition that I did find; in all of the twenty nests examined, only one was found containing one drop of this material; this was in an empty cell of a nest which contained five cells with larvæ, one with an egg, and five empty cells.

Investigation No. 10.—This paragraph relates merely the accidental discovery of honey in a nest of *P. variatus*. This nest was in an inverted, rusty teakettle in a city dump-lot. My attention was attracted to it by seeing the mother enter through a crack near the bottom, where she was obliged to alight and creep under the vessel. By carefully lifting the whole outfit I could observe the contents. The nest was a strong one of about twenty cells with eggs and larvæ. Here *P. variatus* had again demonstrated her fondness for low situations by building her nest in this unusual site near the earth.

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[1] Bouvier ("Psychic Life of Insects," p. 335) says that the social insects have developed from solitary ancestors.

On the morning of May 17, I surprised the mother by lifting up the old teakettle to the level of my eye for examination. She was for a time too much bewildered to move—and the sight that met my eyes affected me likewise, so it was some time before I recovered myself to set it down again. Each one of the eight cells containing eggs had also a large globule of this shining, transparent liquid, so large that it half filled the cell. The next morning, upon similar examination, I found the globules much reduced in size and more viscous in nature. The mother was then on the nest, where I had evidently caught her in the act of bringing in more of it, for in her jaws was a large, shining globule, surprisingly clear at this stage. Three days later these large, watery globules were still present. The mother was again on the nest, but this time she was chewing a yellow ball of caterpillar meat. This was undoubtedly her own food, since I could see no larvæ in the cells to which she could feed it. Thus the evidence indicates that from the very first, *P. variatus* stores a sugary liquid, which with evaporation condenses into a jelly-like substance; that she adds to the store from time to time, and eventually the residue is in the form of sticky balls or drops. It is interesting also that for her own food the queen sometimes catches caterpillars and brings them home to her nest to devour them, as a variation from her nectar diet.

Two weeks later these cells were again seen to contain large globules of liquid, like huge, yellow dew-drops. The eggs had all hatched; the larvæ were still tiny. This set me to wondering whether, in a large, dry field where there was no possibility of getting water, the mother did not gather drops of dew each morning and store her supply of drink for the day. The next day I was greatly disturbed to find that the ubiquitous small boys, who had been spying on my strange behavior in the dump-lot, had discovered the quaint nest and destroyed it.

Investigation No. 11.—At the end of May, 1921, only a few small drops of honey were found in a large number of nests, to be exact, only three in twenty-seven nests. In May, 1922, extensive examinations showed that this year the honey content was so abundant as to be very conspicuous in a large proportion of the nests; twenty-five of the thirty nests each contained the drops in many cells, and larger than usual in size. These were only in the egg cells and the new cells; none were in the cells with larvæ. Just in what way this year afforded conditions which produced this excess, I can only surmise. It does seem probable that the abundance of flowers or the climatic conditions would be factors influencing the supply. On cold, rainy days the queen seldom leaves the nest.

Glancing at the data as a whole one sees at once that this honey-storing habit is much more common in these three species of *Polistes* than was generally thought to be the case. Furthermore, it is probably not a local condition, since the collections from two places thirty miles apart showed this characteristic about equally well established.

Early in the course of this investigation I thought that this substance was the product of the large larvæ. I had observed the workers gathering the saliva-like secretion in large amounts from the mouths of the larvæ, and knew that the adult wasps seemed to regard this as a delicacy and enjoyed it as food. So, since the color was similar, I suspected that the worker, after the material had perhaps undergone some chemical change in their gullets, would place it in the cells, so that the new-born waspling would find a supply of the most delicate food for its first meal.

Soon the evidence from the observations began to militate against this theory. The results of the third group of observations left my belief well-nigh untenable, since the groups of adults whose nests were taken away built clusters of new cells at once, and within three days had the majority of these tiny cells well supplied with this substance. This was done after all their own large larvæ had been carried away. Of course the possibility remains that the adult workers, of which there were plenty at that time, might already have had their crops or gullets full of this, gathered from the nestlings when the catastrophe came, and that they retained this until they could deposit it in the new cells, but this supposition seems far-fetched. Also in Investigation No. 2, nest "*d*," we find a nest entirely without larvæ, yet twenty-five of the thirty cells of the egg stage contained honey. Surely the workers would not venture to go to other nests and rob the larvæ of their salivary secretion; moreover the queens had been storing it at a time when there were no larvæ from which they could gather it. In consideration of these points I felt compelled to doubt or even abandon the idea, and turn to the hypothesis that these drops are vegetable matter, gathered from the flowers and fed to the tiny larva or stored in the cells awaiting its use, like the honey stores of other Hymenoptera. It is unfortunate that we must thus abstractly speculate—that no one has yet actually observed the wasps collecting, making or storing this material, so that we may know with certainty just how it is done and may figure out the relation of the habit to the life or even to the history of the insect. Here, however, I can only continue to give the evidence derived from the foregoing data, in the hope that it may pave the way to conclusive observations in the near future.

In several of the nests observed, especially the new nests described in the third lot, we have in all probability a very definite clue to the nature of the material in these drops. In all nine nests in the third lot, which we knew to be very new, and in other nests where there was evidence that the cells had been made and stored very recently, the globules were exceptionally large and of very watery consistency. This gave rise to the idea that the material is probably similar to that gathered by bees and that it must likewise be subjected to evaporation, as is the nectar in the bee-hive, after which it retains the form of little, firm beads. In the large nests the comparative age or the chronological order of the cells could not be definitely determined, but one small, new nest lent its evidence to this theory. The three central cells each contained a drop, one of them condensed and bead-like, the two others in an intermediate condition. The last cell, a new and incomplete one, contained a huge drop of very watery substance. I placed the entire nest in a tiny tin box to take it home for study, but the large drop was scattered or absorbed by the paper walls en route. This little nest seemed thus to show the natural evolution of a drop of this gelatinous material, from big, watery sweetish drops in the newest cell to smaller, waxy beads in the older cells.

Thus the material from distinct localities shows that this honey is used much more generally than investigators have heretofore suspected. Furthermore, it is present not only at the end of the season, provided to tide over some tardy larvæ, but at all times throughout the season, less frequently during the spring when the queen alone tends the nest, and most frequently and in greater abundance toward the end of the summer. Whether this greater abundance in August is due to a larger supply of flowering plants, or possibly to the greater leisure and number of the workers, or whether they actually gather this material to feed certain larvæ in order to develop queens, remains for further investigation to determine. The frequent lack of this food in the earliest cells of the season might point toward this latter suggestion.

In concluding one would say of Wheeler's theory, that the honey-gathering habit in *Polistes* is a vestigial instinct which is called into play to carry over tardy larvæ, can hardly apply to *P. pallipes*, *P. annularis* and *P. variatus*, since in these species the drops of honey are to be found at times throughout the season. The evidence seems to show that this instinct is not vestigial but functional, and is probably necessary for the survival of the young.

The watery consistency of the drops might give a clue to a solution of the evolution of the habit. We know *Polistes* are heavy drinkers, and probably they give water to the young, as do honey-bees. It might happen that for convenience this water was disgorged on the walls of the cells; by and by a portion of nectar from their throats was accidentally added, and then with evaporation there remained but a small, transparent globule.

Sharp says,[1] in speaking of the habits of social wasps; ". . . the eggs soon hatch and produce larvæ that grow rapidly; the labors of the queen wasp are chiefly directed to feeding the young. *At first she supplies them with saccharine matter,* which she procures from flowers or fruits, but *soon gives them a stronger diet of insect meat.* . . . The hornet is particularly fond of the honey-bee." Unfortunately Sharp does not give us the specific name of the wasp upon which he bases this statement, but that this is based careful observations on some species and not upon mere theory is corroborated by my notes on *Polistes*.

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[1] "Insects," Pt. II., p. 84. [Italics are mine.]

To theorize upon the phylogenetic position of this habit is a small task, and evidently can be substantiated by direct observation. It seems probable that the flower-loving insects evolved from non-flower-frequenting species about the time of the appearance of flower-bearing vegetation. The flower-visiting Aculeates, the bees, were pollen- and nectar-gatherers. If we assume that the bees and wasps had, 'way back in prehistoric time, the same ancestry, we can gather that, phylogenetically speaking, honey-gathering is an older trait in Aculeates than insect-gathering.

Here we have wasps that gather nectar for their own food and honey for their young, and super-imposed upon these traits we have the newer habit of insect gathering for the young. This honey-gathering is not a recent addition to their feeding instincts, but a vestige which proves their ancestry from the bees.[1]

A step further was made when probably certain bee-like, honey-feeding Aculeates back in geologic time, from scarcity of flower food or drought, or through pure "change of mind" or by accident, began to prey upon other insects that sought the same flowers, for their own food or for food for the young in their nests. Sharp tells us that the hornet is particularly fond of honey-bees, which suggests the thought to my mind that the change in habit from the use of flower products to meat may not have been so wide and abrupt as the lines would indicate. It seems so easily possible for certain ones to get a little additional food by licking one another's faces and mouths, just as we see *Polistes rubiginosus* doing today;[2] in time of scarcity of food this habit might easily be exaggerated even to the point of robbery or squeezing out the sweet liquid from the honey-crops, as Fabre describes in the modern behavior of *Philanthus*. From this stage it would indeed be a poor Aculeate that would not eventually learn to abbreviate the work by making a perforation in the body wall to reach the nectar crop directly (just as Robertson describes for *Odynerus foraminatus* that bites an opening into the base of the flowers of *Pentstemon lævigatus*)[2] for the same purpose, or to carry home an entire insect, making it serve as a vessel to hold the honey, without any further trouble. This puncturing of the living honey-pot would lead to a taste for animal juices which we know now exists in certain Pompilids. Even if the habit of carrying this prey home had not by this time been acquired, it would be but a step to learn to carry it to their young after they had learned to enjoy it themselves.

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[1] I am aware that the opposite view is generally held, for Wheeler (*Scientific Monthly*, 15: 68–69, 1922) says: "The structure and behavior of the Sphecoids and Vespoids show that they must have arisen from what have been called Parasitic Hymenoptera, and the structure of ants and bees shows that they in turn must have arisen from the primitive Sphecoids or Vespoids."

[2] Paper soon to be published.

An additional bit of evidence to indicate that the nectivorous habit probably antedates the carnivorous habit in these insects is that, as Sharp tells us and as we have seen in the observations just recorded, the mother feeds to the new-born young first a nectar compound and later, animal food. If we accept the principle that ontogeny recapitulates phylogeny, it is easy to find significance in the fact that the immature organism passes through the nectar-feeding stage before it arrives at the carnivorous stage. Among the surviving descendants of the primitive flower-loving Aculeates, some have digressed to the extent of finally evolving into carnivorous creatures with certain feeding traits that are vegetative.

In my opinion, the honey as I found it was merely stored honey that had been carried in the crop of the queen or the worker for the purpose of direct feeding into the mouths of the young, and this over supply was deposited for convenience on the cell wall. Since these drops are not present at all times in all cells with eggs, in the nests of the three species of *Polistes*, the evidence lends some merit to the theory that these drops are stored by law of supply and demand: a queen having many young larvæ to care for must needs fill the crops of the young directly from her own gullet and has no surplus to store in drops; on the other hand, when the eggs hatch at intervals, the mother has sufficient time during the period of watchful anticipation to store up a surplus. Another factor in regulating the number and size of the drops is the abundance of flowers, and still another reason for the presence of so much in the latter part of the season may be that then the workers are also on hand to help bring it in.

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[2] In order to get the nectar which she could not ordinarily reach, this wasp cut a hole in one side of the base of the tube with her sharp jaws and inserted her tongue; then she cut a hole in the other side and again inserted her tongue. (*Trans. Acad. Sci., St. Louis,* **5**: 569, 1891.)

I often wonder if the honey-storing habit in the honey-bee was not evolved in the same fashion. Is it not possible that originally nectar was gathered and the regurgitated material fed direct to the larvæ; that finally, when workers became abundant in the colony and more was brought in than could be consumed, they resorted to dumping it into cells without larvæ, and that this may have been the forerunner of the honey-storing habit? A contrasting trend of development may be found in the honey-gathering ants, *Myrmecocystus horti-deorum,* which do not eject this substance, and their distended abdomens as they hang to the roof of the honey-chamber are the actual store-houses of the honey.

The reader must bear in mind that the foregoing explanations of this phenomenon are only speculative, but I have tried to use as much logic as I could instil into the ideas. In this I take refuge under the justification of C. H. Eigenmann, when he says: "The imagination is in Biology as elsewhere the guiding spirit. The trouble is our imaginations are sometimes too heavily loaded with statistics, and at other times they fly without the balancing kite's tail of facts. The Palæontologists have contributed to speculative Zoölogy because their imaginations have been kept alive by bridging their numerous gaps, and because they have not been hampered by too great a wealth of material."

ENDODERMAL FLAGELLA OF *HYDRA OLIGACTIS* PALLAS.

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The subject of flagella upon the endodermal cells of hydra was first opened prior to 1871 by Hatchek, who figured a single tapering flagellum upon each cell, all of which he considered similar. F. E. Schulze ('71), figured a single flagellum to each cell of an optical section of a tentacle. Kleinenberg ('72) in transverse sections of the living animal observed one or more flagella in connection with several cells and claimed that the flagella were not fixed structures but that they could be protruded and retracted again, while at the same time the cells sent out pseudopodial processes. T. J. Parker ('80) saw one, two, or three cilia on cell after cell. Because of this observation he drew the inference that the endoderm was ciliated throughout. K. C. Schneider ('90) distinguished at least six types of endodermal cells; but he stated that only three of these types bore flagella. The digestive or epithelio-muscular cells usually bear two flagella, which project into the enteron. The glandular cells bear two or three flagella. The sensory cells bear one, two or no flagella. Schneider gave us, therefore, the first proper description of the flagellated condition of hydra's endoderm. Hadzi ('09) did not carry the knowledge of the flagellated condition of the endoderm beyond Schneider. I have been able to corroborate the work of Schneider and to carry his observations a step further by the use of the following method.

Hydra oligactis was macerated by Mundie's ('26) method. In this case a hydra was placed upon a slide and the water drawn off until but a film remained covering the polyp. The slide was then placed over the mouth of a bottle containing Looper's fixing fluid (made up of equal parts of 95 per cent. alcohol, glacial acetic acid and 40 per cent. formalin [1]). At the end of eight or ten minutes the polyp was rinsed in one or two drops of water, as much of this water as possible drawn off and a drop of Gramm's iodine solution added. The polyp was teased into fragments with needles, a drop of 40 per cent. glycerine added and a coverglass applied. The fragments were then examined under the oil immerson objective. Being in glycerine, the cells could be preserved for a relatively long time, a month or more. During this time the flagella persisted and the cells did not deteriorate. The tissues may even be further treated. For example, if the iodine fades, more iodine may be drawn beneath the coverglass. I have also carried one per cent eosin-licht gruen (95 per cent. alcoholic) solution beneath the coverglass and have, in this manner, stained nuclei, food vacuoles, flagella and other details well. In passing it may be stated that the use of the licht-gruen solution brings out conspicuously the pseudopodia of the epithelio-muscular cells of the endoderm.

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[1] This method was developed in this laboratory by Dr. J. B. Looper.

The epithelio-muscular cells are columnar. A myoneme runs at right angles to the polyp's axis through their broad bases. The distal end bears one or more flagella. There are many food vacuoles in epithelio-muscular cells from well-fed specimens. The secreting cells of the general endoderm are club shaped with the smaller end directed toward the mesoglea. The distal end bears one or two flagella. The distal half of the cell is much vacuolated and in well-fed specimens these vacuoles contain darkly staining material called by Schneider, "Sekretballen." The basal end is darkly granular and bears no myoneme. The sensory cells are of the tall columnar type—almost filamentous. Each contains an ellipsoidal nucleus. These cells bear at their bases slender nodulated processes similar to those figured by Hadzi, in Table II., Fig. 7 and 8. In this type of cell we encountered a flagellum as did Schneider (Fig. 3).

I now come to the point at which my work goes beyond the work of Schneider upon the flagella of hydra. Some of the investigators describe the flagella as being tapering protoplasmic processes. Schneider shows them to be slender and of uniform caliber from base to tip. He does not, however, show a structure that is typically found associated with a flagellum, vis., a blepharoplast. All of my preparations show the flagella of epithelio-mus-

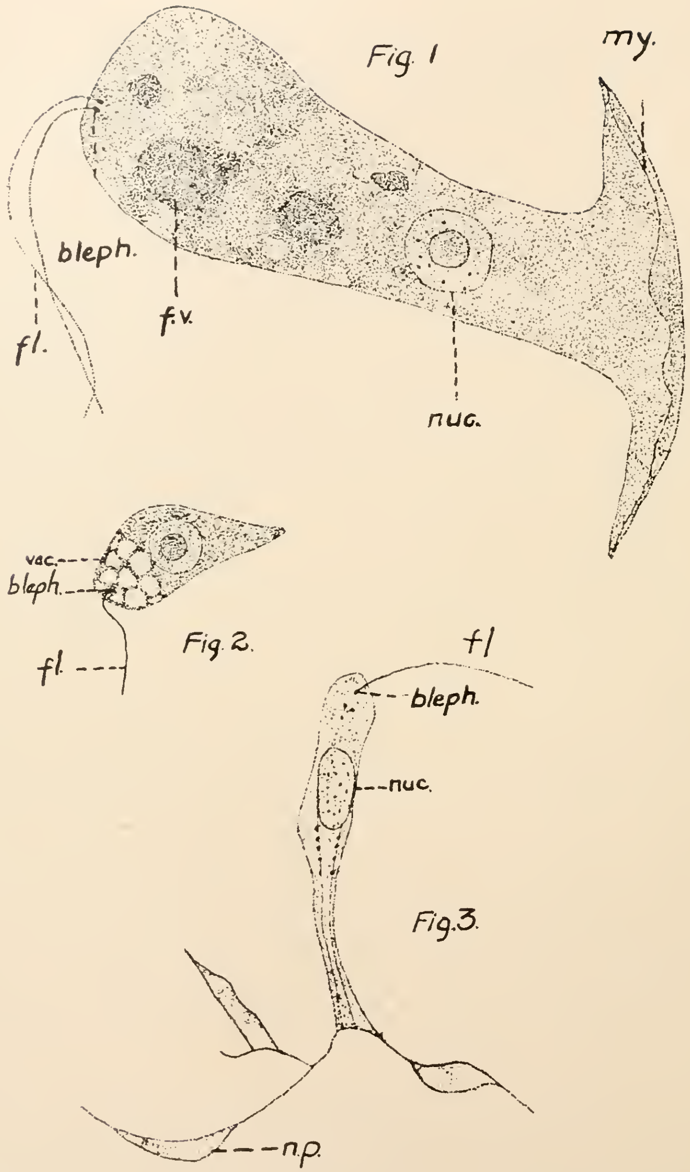
Fig. 1
my.
bleph.
fl.
f.v.
nuc.
vac.
bleph.
fl.
Fig. 2.
fl
bleph.
nuc.
Fig. 3.
n.p.

FIG. 1. Epithelio-muscular cell; *bleph.*, blepharoplast; *fl.*, flagellum; *fv.*, food-vacuole; *my.*, myoneme; *nuc.*, nucleus. × 1000.

FIG. 2. Secreting cell.

FIG. 3. Sensory cell; *np.*, nodulated process.

cular cells, glandular cells, and sensory cells to be associated with a blepharoplast. My Fig. 1 is of an epithelio-muscular cell that shows two flagella each extending into the cell's cytoplasm and ending upon a blepharoplast (Fig. 1, *bleph*). Fig. 2 shows, likewise, that in a secreting cell the flagellum enters the cytoplasm and ends upon a blepharoplast. Finally Fig. 3 indicates that the flagellum of a sensory cell enters the cytoplasm and terminates in a blepharoplast.

Summary.

The cells of hydra's endoderm—epithelio-muscular, secreting, and sensory—are flagellated. The flagella are typical in that they are non-tapering lash-like processes which terminate in blepharoplasts.

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A NEW HISTOLOGICAL REGION IN *HYDRA OLIGACTIS* PALLAS.

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Three anatomical regions are to be recognized in *Hydra:* (1) the oral end bearing tentacles, peristome and mouth; (2) the middle region; and (3) the basal region. In this oral region Kepner and Hopkins ('24) described, in detail, the peristomal endodermal glands that lie about the mouth. They also demonstrated the presence of sphincters at the bases of the tentacles. These sphincters operate against pressure from the enteron towards the tentacles but not in the reverse direction. The histology of the middle region has recently been intensively studied by Hadzi ('09). In this region he finds three types of cells presented by the endoderm: (1) epitheliomuscular cells, that are capable of ingesting small food-particles; (2) gland-cells, which lay down enzymes upon larger masses of food within the enteron; (3) (3) sensory cells. Hadzi, in this respect followed closely Schneider ('90), ('02) who had seen that each of these types of cells bore one or two flagella. Burch ('28) has carried this one step further in demonstrating that the flagella of the three types of endodermal cells arise from blepharoplasts. Not sufficient attention has been given, as yet, to the peculiar cells that lie laterally disposed in the basal region of *Hydra*. These cells are highly vacuolated and larger in calibre than are the endodermal cells of the middle region. In addition to these features, the endoderm of this region is peculiar because of the absence of gland cells. It is not yet clear to us that these cells bear flagella.

The lateral ectoderm of the basal region of hydra does not differ from that of the middle region. The ectoderm of the basal disc, however, has long been known to be peculiar. Korotneff ('80) wrote "Die Epithel-Muskelzellen des Fusses unterschieden sich von den ubrigen Ectodermzellen. Sie besitzen eine cylindrische Form, enthalten eine stark lichtbrechende Fibrille und zeigen in ihrem oberen Drittel eine gleichfalls stark lichtbrechende mucose Ausscheidung, durch welche die Anheftung des Thieres an fremde

Korper bedingt wird" (s. 165). We have not gotten beyond this early interpretation of the histology of the ectoderm of the basal disc.

With reference to the endoderm of this basal disc, however, we have something to add.

It had been noticed in this laboratory for sometime that hydras are sometimes found at low levels in aquaria with small gas-bubbles attached to the basal discs. Mr. George Dare made a careful series of observations upon isolated specimens and found that this gas was developed at the basal disc of hydra independent of the presence of bacteria or other organisms and of temperature changes. We then undertook the histological study of gas-elaborating specimens. We were able to make longitudinal sections that involved the complete or unbroken wall of the gas-bubble. This was done by gently transferring a hydra, that had a small bubble at its end in a drop of water to a slide. The slide was then placed upon a pinch of salt, that lay upon a block of ice, in such manner that the drop containing the hydra lay immediately over the salt. This resulted in rapid lowering of the temperature of the hydra. When ice crystals began to form about the margin of the water in which the hydra lay, it was flooded with Bouin's fluid. Thus the specimen was fixed and sectioned. The sections show the wall of the gas-bubble to be fixed to the marginal cells of the basal disc (Fig. 1, *g*), and the general or central cells to be in contact with the lumen of the gas-bubble, there being neither secretion material, bacteria, nor other substance lying over their free ends (Fig. 1, *h*). It was thus seen that the wall of the gas-bubble is composed of the mucus-like secretion of the basal disc. The basal ectoderm is, therefore, concerned with the elaboration of both the mucus-like material with which the hydra fixes itself to some submerged surface, and with the elaboration of a gas.

Our chief point of interest, however, lies not in the ectoderm of the basal disc but in the endoderm; for here we find a feature that has not been described. The endodermal cells of the basal region are larger than those of the middle and oral region, and bear great vacuoles. They do not ingest food particles (Fig 1, *e*). In this respect they again differ from the middle and oral regions' endodermal cells. Until recently these cells were supposed to overlie the basal disc's ectoderm Indeed Curtis and Guthrie ('27) have illustrated these peculiar cells as lining the fundus of the enteron. In our gas-secreting specimens we find that the fundus of the enteron is lined with endodermal cells that bear numerous food-vacuoles. They are tall, columnar cells having myonemes in their bases. They resemble the epitheliomuscular cells of the middle region of the body. We have not been able to isolate them for maceration methods and cannot, therefore, say that they bear flagella. But they so closely resemble the epitheliomuscular cells of the middle region that our inference is that they do bear flagella. The presence of these cells, therefore, in the fundus of the enteron gives us an epithelial region that is like that of the oral and middle regions of the body except that there are no gland-cells in this basal endoderm.

The new histological region that we have thus discovered lies in the wall of the basal disc. The ectoderm of this region we have found to conform to the descriptions of earlier investigations; but the endoderm is peculiar in that it is an epithelial disc composed of columnar epitheliomuscular cells, that contain food-vacuoles, and which presents no gland-cells.

The presence of this endodermal disc, that is active in food-appropriation, is of interest when we keep in mind the dual function of the basal disc. The elaboration of gas takes place relatively rapidly. The rapid elaboration of gas would, therefore, involve rapid metabolism. The basal disc, then, is a region of relatively high metabolism. The presence of this food-getting endoderm in this region falls in line with the observation of Tannreuther ('09) when he says that "Those endodermal cells of the region of growth, 'the formation of buds and sexual organs', are the most active in ingesting partly digested food from the enteron and preparing it for diffusion into the ectodermal cells" (p. 211).

The basal disc has, therefore, a peculiar endoderm correlated with the dual function of mucus- and gas-elaboration.

The elaboration of gas is done in order that the specimen may be lifted in the water. The gas is discharged into the sac of mucus until the bubble formed lifts the animal to the surface of the water. As the polyp rises it has its basal end directed up and oral end pending. When the bubble encounters the surface film of water, its wall ruptures and forms a somewhat circular disc of mucus, that is closely applied to the surface film of the water to form a raft by which the polyp hangs (Fig. 2, *g*). Wave-action will destroy this raft and then the specimen will sink. Or the hydra may abstrict itself from the raft of mucus and sink.

SUMMARY.

We have observed that the basal disc's ectoderm in hydra not only secretes adhesive material, with which the polyp fixes itself to some submerged surface, but that it also under certain conditions elaborates a gas. This gas is caught within the mucus-like secretion of the basal disc and retained therein. The bubble, thus retained, increases in size until the hydra is lifted to the surface by it. At the surface of the water the retaining vesicle of mucus breaks and spreads as a circular raft from which the hydra hangs beneath the water's surface.

The ectoderm of the basal disc is thus seen to have a double function. Associated with this region of the ectoderm of hydra there has been found a pecular region of endoderm. The endoderm of this region is characterized by its component cells having the appearance of the epithelio-muscular cells of the oral two thirds of the body as over against the highly vacuolated epitheliomuscular cells of the aboral third of the body.

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EXPLANATION OF PLATE.

FIG. 1. Longitudinal, axial section of hydra. *a*, peristomal gland-cells of endoderm; *b*, gland-cell of middle region's endoderm; *c*, epitheliomuscular cell of endoderm, bearing two flagella and two food-vacuoles; *d*, epitheliomuscular cell of endoderm ingesting a food particle; *e*, epitheliomuscular cells of lateral endoderm of basal region; *f*, endoderm of basal disc; *g*, mucus-wall of the gas bubble; *h*, basal disc's ectoderm that is exposed to lumen of gas bubble; *i*, ovary; *k*, testes.

FIG. 2. Basal disc of polyp at surface film of water. *s*, surface film of water; *g′g′*, circular raft formed by ruptured wall of gas bubble now applied to surface-film of water; *h′*, ectoderm of basal disc that is now in contact with air.

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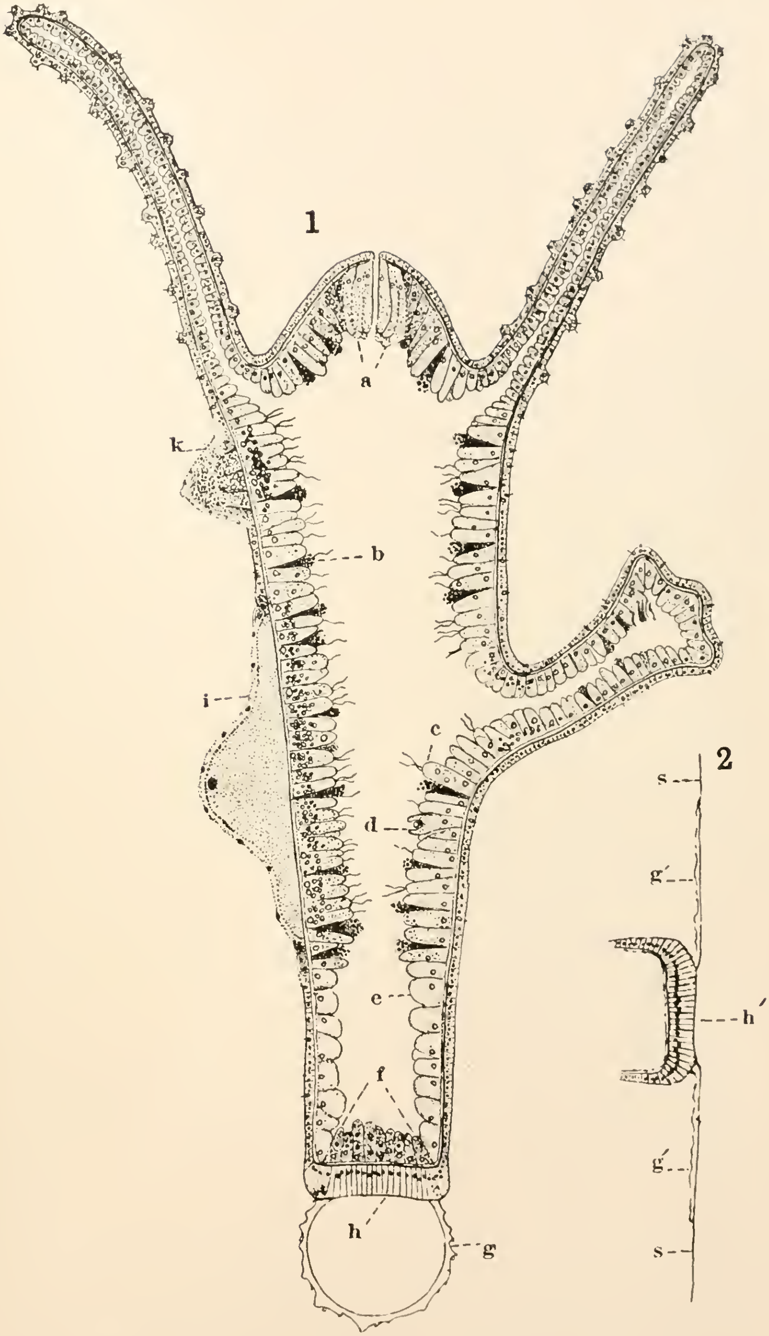
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WM. A KEPNER AND LULU MILLER.

HISTOLOGICAL FEATURES CORRELATED WITH GAS SECRETION IN *HYDRA OLIGACTIS* PALLAS.

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The ectoderm of the basal disc of hydra ordinarily elaborates mucus by means of which the polyp adheres to some submerged surface. It has recently been observed that this same ectoderm may, under certain circumstances, elaborate gas. This gas is held within a sack of mucus (Kepner and Miller, '28). This fact has suggested to us that there may be some interesting histological change correlated with the elaboration of gas. Kepner and Miller have found, to begin with, that there is a peculiar region in hydra's endoderm in the basal disc. This endoderm supplies the food demanded by the metabolism that yields the two products of the basal disc's ectoderm—mucus and gas.

Their observation concerning this local accumulation of food was interesting; for it carried the point, arising out of Tannreuther's ('09) observation, a step further. Tannreuther's observation was that in the region of a developing gonad or of an incipient bud there were local and pronounced accumulations of food within the endoderm. Yoder ('26) found that there was a marked accumulation of glycogen in these regions of most active metabolism. Finally Kepner and Hopkins ('24) observed that there was not a wide diffusion of material absorbed by the endoderm. For example, they recorded that "there is no extensive diffusion of absorbed chloretone through the tissues of the body. A diploblastic animal, therefore, cannot possess anything comparable to a circulatory medium" (p. 448). Our observations further strengthen the hypothesis that local needs must be met locally.

The detailed histology of the basal third of a hydra, that had not been secreting gas, shows the lateral endodermal cells to be stout and highly vacuolated (Fig. 1, *e*). The extent of distribution of these lateral cells varies greatly in different specimens and perhaps also in the same specimen at different phases of the polyp's activity. The cells of the endoderm of the basal disc are more slender and present a denser cytoplasm than do the cells of the above lateral endoderm. They also carry within their cytoplasm food vacuoles (Fig. 1, *f*). Food-vacuoles are not present in the lateral endoderm of the basal part of hydra. The lateral ectoderm of the basal third does not in any manner differ from that of the general ectoderm of the body. The ectoderm of the basal disc, however, presents features that are peculiar to it. In the first place, there are no nematocysts in this region, of the outer epithelium. These cells, moreover, when actively discharging mucus have conspicuous inclusions within their cytoplasmic bodies. These may be designated secretion-granules (Fig. 1, *m g*). These cells extend over the entire basal disc. All attached hydras will not show the secretion-granules in the ectodermal cells throughout the extent of the basal disc. A specimen that has long been attached to the substratum may not yield, when fixed and stained, sections that display secretion-granules. Only specimens caught at the time they are fixing themselves to a substratum will present

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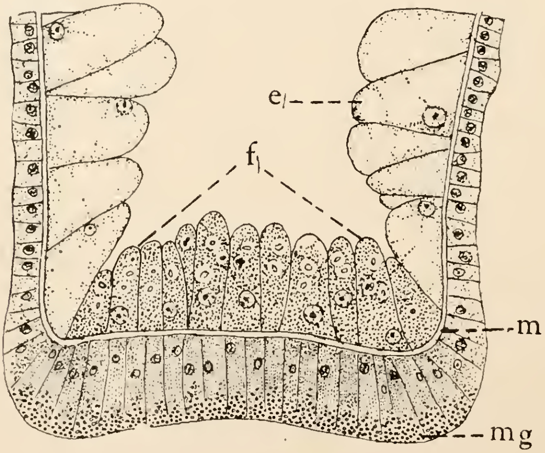

Fig. 1. Partly diagrammatic, axial section of base of hydra that had been elaborating mucus in order to fix itself to substratum. *e,* lateral endoderm of basal third of body; *f,* peculiar endoderm that lines the fundus of the enteron and contains food-vacuoles; *m,* mesoglea; *mg,* mucus-granules arising within the mucus-secreting cells of ectoderm. × 650.

these secretion-granules in sections (Fig. 1, *m g*). The most emphasis must here be placed upon the condition of the mesoglea. In a mucus-secreting specimen, the mesoglea of the basal disc differs in no manner from that of the body proper. It presents a uniform, unbroken contour (Fig. 1, *m*). All this in no manner presents anything new concerning the histology of hydra.

In the gas-secreting hydra some conspicuous histological features appear that have not been recorded. In the first place, the endodermal cells of the basal disc appear to be larger and more active in the gas-secreting specimens than in the non-secreting ones. Next we find that the ectodermal cells of a wide peripheral region of the disc elaborate mucus. These, in other words, do not have their usual function changed. The axial cells in the basal disc's ectoderm, however, do have their function altered. They no longer present secretion-granules and therefore stain (in hæmatoxylin) less than do the peripheral cells of the disc. We have now an epithelium the periphery of which elaborates a retaining wall of mucus, while the axial region secretes gas into the mucus to form a buoy or lifting float for the polyp (Fig. 2, *mc* and *gc*). The most conspicuous feature of the gas-secreting polyp lies in the mesoglea's modification. The mesoglea, in this disc at this time, becomes greatly swollen and highly vacuolated as though the endoderm had flooded it with a deposit of metabolic substance. Within this broken region of the mesoglea there appears, in fixed material, a substance that suggests a coagulated plasma (Fig. 2, *m'*). The presence of this plasma within the mesoglea may signify one of two things: (1) It may be that food is being deposited there by the endoderm in order to meet the demands of an intense metabolism taking place during gas-elaboration, or (2) It may be that metabolic substances are being dammed back from the relatively active axial ectoderm while it is elaborating gas. If the first alternative be correct, a plasma, as it were, is thrown down locally into the axial mesoglea of the basal disc in order that the gas-secreting cells may be abundantly supplied with food during the peculiar metabolism involved. If the second alternative be correct, it means that the axial ectoderm is throwing metabolic wastes into the mesoglea. Ordinarily the ectoderm discharges its metabolic wastes externally through a moist mucus when the polyp is fixed, or directly into the water when the polyp lies free. When, however, a layer of gas is deposited upon the outside of this basal, axial ectoderm the osmotic drainage is blocked. The presence of the gas no longer lets the metabolic wastes drain by means of an osmotic exchange through the free ends of the axial, ectodermal cells. Hence metabolic wastes, instead of metabolic food, back into the mesoglea to flood it and form the vesicle that we have observed. As the elaboration of gas advances the vesicle of the mesoglea, together with its included plasma, decreases until, at the time of the gas-buoy's attaining its maximum size, the mesoglea has returned to the condition characteristic of the general mesoglea.

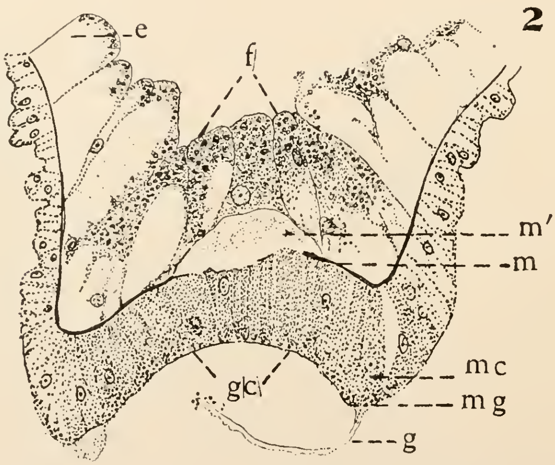

FIG. 2. Axial section of base of hydra that had been elaborating a gas-bubble. *e,* lateral endoderm of basal third of body; *f*, peculiar endoderm that lines fundus of enteron and contains food-vacuoles; *gc*, gas-secreting region of ectoderm; *mg*, mucus-granules; *g*, mucus wall of gas-bubble; *mc*, mucus-secreting region of ectoderm; *m* unbroken, but deeply staining region of mesoglea; *m′*, plasma-like material within distended region of basal mesoglea. × 650.

It matters not which of the above alternatives be the correct interpretation, the interesting point may be made that, in the basal disc of hydra secreting gas, we have a situation arising that makes a peculiar demand upon the passive mesoglea. As a result, the mesoglea is sometimes flooded either with metabolic food or with metabolic wastes. Thus we have the mesoglea foreshadowing the function of a true circulatory medium such as is found in the plasma of the Turbellaria or even in that of the blood and lymph of the higher triploblastic animals.

SUMMARY.

The basal disc of hydra has a two-fold secretory function: (1) It secretes a mucus by means of which the polyp is anchored. (2) It secretes gas that is retained within a wall of mucus by means of which the polyp is lifted to the surface of the water. This dual function of the basal disc places a peculiar metabolic demand upon the disc's endoderm, ectoderm and mesoglea. As a result of this peculiar metabolism and the conditions arising out of gas-secretion, the mesoglea becomes flooded with a plasma and thus handles either metabolic food or metabolic wastes in a manner that foreshadows the plasma of the circulatory media of triploblastic animals.

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THE LIGHT-RECEPTIVE ORGANS OF CERTAIN BARNACLES.[1]

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INTRODUCTION.

Although brief references to the light-perceptive organs of barnacles are found throughout the literature, no really comprehensive study has been made of them since that by Pouchet and Jobert in 1876. The relationships between the "eyes" in the larval stages and those in the adult have never been fully worked out. Accordingly the object of this investigation has been a study of the various light-perceptive organs found in the larval and adult barnacles and the determination of their relationships.

Investigations relating to the light-receptors of barnacles are usually found combined either with studies of other structures of the barnacle or with studies of the eyes of Crustacea in general. Among the former type Darwin's "Monograph of the Cirripedia" (1851 and 1854) is outstanding. More recently Gruvel (1905) has published a detailed account of the morphology and taxonomy of this group. Grenacher (1879), Claus (1891), Brooks and Herrick (1892), and Demoll (1917) have studied the eyes of Crustacea. General problems in this connection have been taken up by Parson (1831), Patten (1886 and 1887), and Mark (1887).

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[1] The author is indebted to Dr. J. P. Visscher of Western Reserve University for the suggestion of the problem and for assistance during the course of the investigation.

Materials and Methods.

The ivory barnacle, *Balanus eburneus,* and the rock barnacle, Balanus balanoides, were used in this study. Collections were made at Woods Hole, Mass., during the spring and summer of 1926. Living forms were observed at this time and material was preserved for subsequent study.

Adults of *Balanus eburneus* brought to the laboratory often contained Nauplei nearly ready for hatching. Unhatched Nauplei were obtained by removing the ovigerous lamellæ from these adults, and free swimming forms were collected after hatching. A few metanauplei and Cyprids were obtained from towings made in the vicinity.

In order to test the actual light-perceptive function of the adult, the shell was broken so that the eyes could be exposed. Specimens with the shell thus broken were tested to make sure that the light reaction was unimpaired. The eyes were then removed by a hot needle. In all cases included in the data, the eye adhered to the needle and was removed without apparent injury to the surrounding tissue.

Material was fixed in picro-acetic formol (Bouin's) or in 10 per cent. formalin, and was either stained in Ehrlich's hematoxylin or mounted unstained. Most of the study was done by means of sections which were prepared by the ordinary paraffin method and cut from 5μ to 10μ in thickness. Grenacher's technique for the removal of pigment (Lee, 1890) was used on sections of the Cyprids. In addition a modification of Cajal's silver nitrate technique as developed by Hess (1925) was used in the study of the adult eye.

The Nauplian Stage.

The formation of the median eye in Cirripedes has never been worked out. In *Balanus eburneus* the eye first appears in the unhatched Nauplius as an elongated area of reddish pigment. Certain authors, for example Darwin (1851), have believed that the nauplian eye might arise from the union of two anlagen but, at least in *Balanus eburneus,* this does not appear to be the case. A large number of specimens in which the eye was just forming were studied but in all cases it appeared as a single area with no evidence of a double origin. In succeeding stages the pigment becomes darker and the eye appears as a bilobed structure.

The morphology of the nauplian eye of Cirripedes has usually been treated incidentally in connection with studies of the complete animal and consequently its detailed structure is known in only a few forms, chiefly among the Lepadæ. The pigmented area in the anterior region of the Nauplius was noticed by the earliest authors but their descriptions mention only the general shape of the pigment cup. A fairly complete study of the light receptors in the Cirripedia was made by Pouchet and Jobert in 1876. These authors described the nauplian eye as formed of two parts each of which represented a simple eye and was composed of a pigmented body with finely granular, rose-colored pigment and small oval bodies which stained black with osmic acid and which they thought might be designated as lenses, although the analogy was somewhat questionable. The median eye of Crustacea in general was described by Demoll (1917) as an inverted eye composed of two pigment cells forming a cup in which were the visual cells which extended proximally in a nerve fibre. These visual cells were in the nature of "Stifchensäume" and the whole eye cup was lined with fine, reflecting scales which formed the "tapetum." A cellular lens was said to be present in some forms.

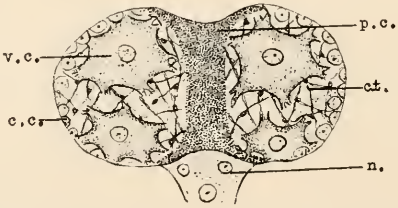

FIG. 1. Diagrammatic drawing of the median eye in the cyprid stage (longitudinal vertical section × 500). *c.c.*, cortical cells; *c.t.*, connective tissue; *n.*, nerve; *p.c.*, pigmented cells; *v.c.* visual cells.

When sections of fully developed Nauplei are studied the median eye (Fig. 1) can be seen lying between the so-called "cerebral ganglia." The eye at this period measures about 15μ in diameter and, due to this small size, an interpretation of its structure is very difficult. The central region is composed of heavily pigmented cells which form a bi-concave pigmented cup. In each concavity there is a non-pigmented area coated by cells with large nuclei, similar to ones which form the cortical area of the cerebral ganglia. The non-pigmented area appears to be made up of two visual cells surrounded by a small amount of connective tissue. Each area sends off a nerve which is probably formed by an extension of these visual cells as in the adult. These nerves leave the posterior region of the eye and by very short connectives enter that part of the ganglia which is slightly posterior to the eye.

A lens as described by Claparède (1863) for *Lepas* is not present in *Balanus*. The non-pigmented area composed of visual cells and connective tissue was probably mistaken for a lens by Pouchet and Jobert (1876). This description is not in accord with that given for the median eye of other Crustacea by such authors as Hesse (1901), and Demoll (1917). The eyes of the two Cirripedes studied show ganglion-like visual cells instead of the " Stabchen " or " Stiftchensäume " described by these authors. While it is difficult to determine accurately the morphology of the median eye at this period, the structure, as described, agrees fully with that observed later in development. Hanström (1927) in a recent article has described a somewhat similar structure in the larval eye of *Nymphon stromi*. Whether the lack of agreement concerning the structure is due to error on the part of the foregoing authors or whether there is a real difference in the median eye of these Cirripedia is a point which has not yet been determined.

In addition to the median eye characteristic of the Nauplius, the Metanauplius of *Balanus eburneus* has a pair of compound eyes which are generally known as the cyprid eyes since they are the most noticeable light-perceptive organs of that stage. The cyprid eyes in the Metanauplius appear on either side of the median eye near the base of each of the first pair of nauplian appendages. Their structure at this period is superficially the same as in the Cyprid but material was not available for histological verification of the appearance. The nauplian eye retains its characteristic position and appearance during the metanauplian stage.

In common with the Nauplei of other barnacles those of *Balanus* *eburneus* show definite reactions toward light. In a dish containing Nauplei these were ordinarily found congregated in the area having the greatest illumination. Occasionally a few individuals were observed which collected at a point just opposite this brightest spot. The reason for this variation has not been determined but it seems to be constant in such specimens and therefore is not the same type of reaction as that described by Groom and Loeb (1891) in which the same individuals responded differently under different conditions.

The Cyprid Stage.

The early authors had not observed the Metanauplius stage of Cirripedia and so did not always clearly recognize the distinction between the median and compound eyes. Burmeister (1834) described the median eye as a rounded, black spot which became divided and modified to form the paired, compound eyes of the Cyprid. This error persisted through several of the later works and Darwin (1854) reported Burmeister's observation although he considered it " scarcely possible that the eye of the larva of the first stage can be changed into the double eyes of the second stage." It was not until the two types of eyes were observed present at the same time that this misapprehension was fully removed.

The fate of the compound eyes at metamorphosis into the adult has been variously described. Darwin (1851) and Hesse (1874) noted that the eyes fell from their capsules during metamorphosis while Von Willemoes-Suhm (1876) stated that before metamorphosis the eyes lost their original position and might be seen only as black pigment spots which were later absorbed. A similar observation has been made by Coar[1] in an unpublished study of *Balanus balanoides* and by Hanström (1927). The author has observed the extruded eyes in slides of *Balanus amphitrite* collected by Dr. J. P. Visscher at Beaufort, North Carolina. However, the condition in *Balanus eburneus* seems to be similar to that described by Von Willemoes-Suhm and Coar. The two late cyprid stages which were observed at metamorphosis showed no compound eyes, the region of these being occupied by pigment masses which may have been the degenerating eyes.

The compound eye, as seen in total mounts, has been described by all authors studying the Cyprid. In such preparations the eye appeared as consisting of a black pigment body and eight to ten globules or lenses surrounded by a large capsule, and is described as such by Darwin (1851), Hesse (1874), and Von Willemoes-Suhm (1876). When studied in section the compound eyes of *Balanus balanoides* (Fig. 2) are found to be situated in pockets near the bases of the antennules. In contrast to the rather unique structure of the median eye, their appearance is very similar to that of the compound eyes of other Crustacea. The eight to ten lenses described for similar forms by early authors are present and represent the same number of visual elements or ommatidia. Each ommatidium contains a cuticular lens surrounded by "corneagen cells," which are reported by Patten (1886 and 1887) to secrete this body, and other cells known as the cells of the crystalline body. The lens or crystalline body is made up of three units forming an egg-shaped structure. The proximal portion of the ommatidium contains the rhabdomes surrounded by heavily pigmented retinular cells. This region is much shorter in the compound eyes of Balanus than in most Crustacea. It was not possible to study the rhabdome in detail but it appears to be like that of other Crustacea, of reticular nature and penetrated by nerve fibrils. The retinular cells pass into the optic nerve of the com-

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[1] Personal correspondence, 1926.

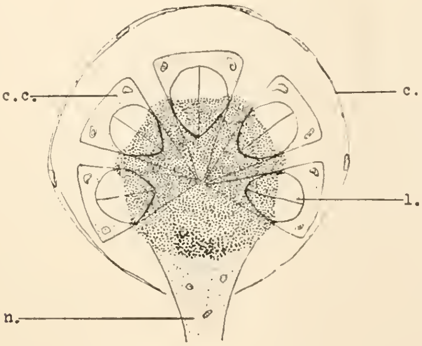

FIG. 2. Diagrammatic drawing of the compound eye in the cyprid stage (vertical section × 300). *c.*, cornea; *c.c.* corneagen cells; *l.*, lens; *n.*, nerve.

pound eye. The ommatidia are surrounded by a common corneal sheath. The structure of the compound eye of *Balanus eburneus* appears to be similar, as far as could be determined from a study of total mounts.

In 1851, Bate noted that the pigmented area of the Nauplius was represented by a similar region in the Cyprid although he did not believe that the area was a light-receptor in either period. Claus (1869), in a drawing of an unknown Cyprid, pictured the persistence of the unpaired eye up to the moment of metamorphosis, although the eye is not labelled and it is probable that he did not realize the significance of his observation. Pouchet and Jobert (1876) recognized and discussed this persistence of the median eye through the cyprid stage.

The median, or nauplian, eye (Fig. 1) persists throughout the cyprid period. It has enlarged to five times its former diameter (15μ–79μ) but its structure is the same. The cells with large nuclei still coat the non-pigmented areas and because of the similarity of these nuclei to those of the regular visual cells, it is extremely difficult to determine the exact number of the latter. The bi-concave pigmented area is composed of many cells in contrast to the condition reported for *Lepas,* where only two cells are found. In each pigmented area there are two non-pigmented zones each containing two visual cells which form a nerve connection with the cerebral ganglia. These nerve connections have elongated while the amount of connective tissue surrounding the visual cells has also become greater and the reticular nature of part of it is evident in most sections.

It is difficult to say just what part the compound and median eyes play in the light perception of the Cyprid. Probably both are functional. Since the median eye is functional in the stages preceeding and following this period, as well as after the degeneration of the compound eyes, it is unlikely that it would completely lose its function at this time.

THE ADULT STAGE.

Early observers denied the presence of an eye in adult barnacles and indeed the relatively degenerate structure and enclosing shell of the adult tended to support this view, as well as the fact that all eye structures were thought to be lost at metamorphosis, with the disappearance of the cyprid eyes. The first report on the existence of eyes in the adult barnacle was made by Leidy (1848) on *Balanus rugosus* (sp?). In 1854, Darwin substantiated Leidy's report by finding eyes present also in the adult of *Balanus tintinnabulum.* A summary of previous studies of the barnacle eye was made by Gerstaecker (1866). These were concerned chiefly with the fact of occurrence or with external appearance of the eyes. The most complete study of the barnacle eye in the adult was that made by Pouchet and Jobert in 1876.

There seems to be no published report of the origin of the eyes in the adult. Darwin (1854) pointed out that they were not developed from the eyes of the Cyprid, since the new eyes were formed at some distance from the compound, but thought they might have been formed from the nauplian eye since they occupied a similar position. Coar[1] reported that in *Balanus balanoides* the adult eyes were formed by a division of the median or nauplian eye, and the author has found that the same situation occurs in *Balanus eburneus.* The eye of the Nauplius, which has persisted throughout the cyprid stage, divides into two parts during the metamorphosis of the Cyprid into the adult. These, together with a part of the mantle which becomes modified around them, form the simple, paired eyes of the adult. In animals which have just completed metamorphosis the two eyes may be seen completely separate although still very close together (Fig. 3 *A*). They move apart during the succeeding period and, at about the fifth day, are in the position which they occupy in the adult (Fig. 3 *B*). The eyes were found to lie in the mantle between the scutum and the juncture of the rostrum (rostrum coalesced with rostro-lateral-Darwin 1851) and the lateral plates of the shell. Immediately around the eye and optic nerve the mantle lacks its usual pigmentation and for this reason the eye appears prominent. The part of the eye toward the body of the barnacle is heavily pigmented while the region toward the shell is without pigment.

Pouchet and Jobert (1876) described the eye as a rounded structure partly covered by pigment and adherent to the surrounding tissue "en arrière." They believed that this pigment functioned as a choroid coat while the non-pigmented area might possibly be called a cornea. When the eye was macerated in Müller's fluid they noticed the presence of a cell which owing to its volume, granular nature, and distinct nucleolus, they felt was undoubtedly a nerve cell. The existence of a double optic nerve suggested to them the possibility that there were two such cells. However, they never observed more than one and were inclined to consider the situation analogous to that in the Lepadæ where they had found a double nerve but always a single nerve cell. The light was described as reaching the eye by traversing the tissue which united the valves and which contained no pigment in the vicinity of the eye. They found that barnacles were sensitive to light, but did not believe that they possessed object vision. No mention is made of the origin of the adult eye although development from the median eye is suggested by the nature of the remarks.

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[1] Personal correspondence, 1926.

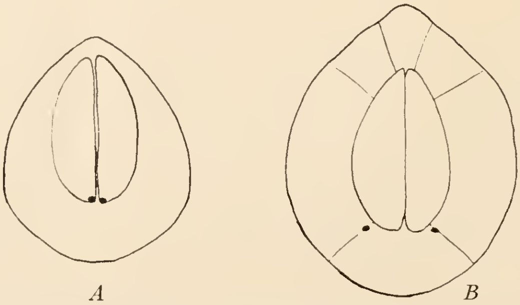

FIG. 3. Diagrammatic drawing showing adult of *Balanus eburneus*. *A*, immediately after metamorphosis; *B*, five days after metamorphosis.

When sections of the eye of the adult are studied it is seen to be composed of two main divisions; an outer covering which is a modification of the mantle and an inner part which is developed from the divided nauplian eye (Fig. 4).

The outer covering is composed of irregularly shaped cells similar in appearance to those of the mantle. Pigmented cells make up about half the area of this coat and are continuous with like cells in the mantle, while non-pigmented cells form the rest of the covering and are a continuation of similar mantle cells.

Between this covering and the inner portion of the eye is a region of loose collagenous connective tissue fibers which serve to hold the inner region in position as well as to support the outer covering. In the living specimen the interstices of these fibers are filled with fluid which helps to maintain the contour of the eye.

The inner part of the eye is a sphere containing pigmented cells, collagenous fibers, reticular fibers, and visual cells with their nerves. The pigmented region forms a cap over about half of this portion of the eye and lies directly beneath the pigmented region of the outer coat. Beneath this, is an area of rather loose connective tissue fibers and cells similar to those between the outer and inner regions. The rest of the inner eye is composed of a reticulum of connective tissue fibres surrounding two large ganglion or visual cells. A slight division is observable in this reticulum, indicating the two units of nervous elements. These two visual cells send off branches which ramify throughout the reticular fibers. Each cell also gives off a nerve fiber which is surrounded by a sheath. These fibers, with their individual sheaths, become united as a double nerve enclosed within a common connective tissue coat before passing through the outer covering and continuing to the supracœsophageal ganglion as the optic nerve.

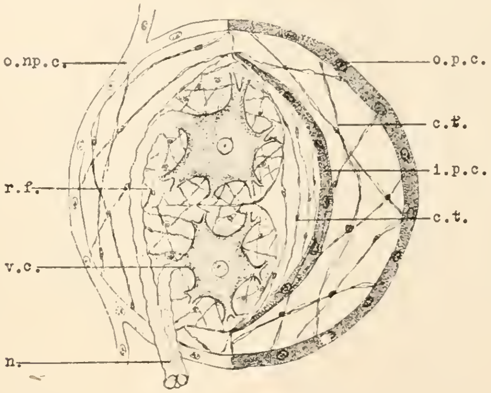

Fig. 4. Diagrammatic drawing of the simple eye of the adult (vertical section × 20). *c.t.*, connective tissue fibres; *i.p.c.*, inner pigmented cells; *n.*, nerve; *o.np.c.*, outer non-pigmented cells; *o.p.c.*, outer pigmented cells; *r.f.*, reticular fibers; *v.c.* visual cells.

The enlargement of the nerve just prior to its entrance into the pigment mass, as described by Gruvel (1905), is not apparent in the sections and it seems probable that he has included the non-pigmented region of the outer coat as a part of the nerve. Since his work did not include studies of sections of the eye, this error is very natural.

Papers on the median eye of crustacea often describe the presence of a tapetum next to the two (or more) cells which form the eye cups. By homology the inner pigment region of the adult barnacle eye corresponds to these cells and the tapetum might therefore be either the loose connective tissue or the reticular regions of this eye. However so little uniformity is found in the use of this term by investigators that it is considered inadvisable to apply the name to any particular region of the barnacle eye.

Barnacles in their natural environment will retract their cirri when stimulated by light, and this fact is mentioned by several of the early authors. Pickering (1848) brought it forward in confirmation of Leidy's report as to the existence of eyes in the adult. Darwin (1854) observed the reaction to light in *Balanus balanoides, Balanus crenatus,* and *Chthamalus stellatus* and found that they were all sensitive to a shadow produced by passing his hand between them and the light. Gerstaecher (1866) reported experiments by Fr. Müller who found that *Balanus tintinnabulum* would react to a shadow when the body was removed and the eyes and certain muscles were left in the shell. (" dass *Balanus tintinnabulum* auf eine Beschattung mit der hand auch dann reagire, wenn er mit Zurücklassung seiner Augen an dem Manteldeckel, von diesem abgelöst werde. Ein in dieser Weise entblösstes, mit halb entrollten Ranken im Wasser liegendes Exemplar zog dieselben jedesmal schnell ein, wenn es beschattet wurde.")

In our study it was found that the adults of both *Balanus balanoides* and *Balanus eburneus* close the opercular valves if there is a sudden change in light intensity although very gradual changes do not excite the reaction. *Balanus eburneus* is more sensitive to such changes than is *Balanus balanoides.* A slight shadow may sometimes be cast upon the latter without affecting them but it was never possible to do this with *Balanus eburneus.*

Since it is shown that the adults possess some light-perceptive mechanism, it becomes necessary to find what part the so-called "eye" plays in causing this reaction. Accordingly, the light reactions were studied in twenty-five adults of *Balanus eburneus* which had been deprived of these organs and it was observed that such animals showed no reaction to light changes, however sudden or intense, although when the eyes were intact they had all withdrawn their cirri and closed the valves under similar conditions. Therefore it is concluded that these organs are the sole light-perceptors present in the adult barnacle.

The function of certain parts of the eye is problematical. Since the ganglion or visual cells send branches throughout the reticulum of connective tissue fibers immediately surrounding them, it is evident that this part serves in the transmission of the impulse. The looser collagenous fibers outside this area do not have any self-evident function. As they are not pigmented they would not prevent the passage of light and it seems probable that their function is merely that of support. The inner pigmented cells may be either protective or reflective in nature. The structure of the eye and its inverted nature lend some support to the latter possibility. The light enters the eye through the non-pigmented area of the outer coat while its entrance through the other cells is prevented by the pigment. The fluid which is found in the eye of living specimens must act as a refractive as well as a supporting medium.

SUMMARY.

1. The development, structure, and function of the light-perceptive organs are described in the nauplian, cyprid, and adult stages of *Balanus eburneus* and *Balanus balanoides*.

2. The light-perceptive organs present in the various stages are: (*a*) nauplian—a median eye, (*b*) metanauplian—a median eye and two compound eyes, (*c*) cyprid—a median eye and two compound eyes, (*d*) adult—two simple eyes.

3. The median eye in *Balanus eburneus* originates as a single, pigmented mass in the unhatched Nauplius and persists with no change, except in size, until the metamorphosis of the Cyprid into the adult.

4. The compound eyes first appear in the metanauplian stage and remain functional throughout the cyprid stage.

5. These compound eyes are resorbed at the time of the metamorphosis of the Cyprid into the adult.

6. At the metamorphosis into the adult the median eye divides into two parts which form the simple paired eyes of the adult.

7. Each of the paired eyes in the adult is the morphological equivalent of half of the median eye plus an outer covering.

8. The simple, paired eyes are the sole light-perceptive organs of the adult.

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